FlowJo

Data Analysis Software for Flow Cytometry

User Documentation
Tutorial

FlowJo was written by Adam Treister and Mario Roederer, based on concepts developed with David Parks, Martin Bigos, and Wayne Moore.

We are indebted to past and present members of the Herzenberg laboratory at Stanford for their ideas, discussions, and tireless testing of new versions.

Mercutio MDEF is Copyright © Ramon M. Felciano 1992-1997.
LMoovController is Copyright © Paul Lalonde 1998.
Various utility code was written by John Norstad © Northwestern University

FlowJo is Copyright © Trustees of Leland Stanford Jr. University, 1996-97
FlowJo is Copyright © Tree Star, Inc. 1997-2001.
FlowJo Tutorial and Web Site are Copyright © Tree Star, Inc. 1997-2001.

Revision Date: 18 April 2001
Version 3.4

FlowJo Tutorial

FlowJo is a software application designed to create an integrated environment for viewing and analyzing flow cytometric data. This environment is presented in the form of a ***Workspace***. The Workspace contains a list of all of the data samples that you load, the gates, statistics and other analyses that you apply, and the table templates and graphical layout templates that you design. The Workspace is saved as a FlowJo document on your hard disk; when you re-open the document, you will see the status of your analyses as they were when you last saved the Workspace.

This tutorial is designed to introduce you to the program. Reading through it, you will learn how to operate FlowJo. Run the program as you perform the steps in the tutorial so that you can get the best feel for how the program works! As you watch FlowJo perform various operations such as creating new graphs, statistics, tables, or graphical layouts, you will see how fast and easy FlowJo is to use. It will take you three to six hours to complete the tutorial (you can easily break it up by chapter).

The tutorial is designed around an example data set. The experiments used for the tutorial are based on 3-color immunophenotyping of human peripheral blood mononuclear cells (PBMC). The steps shown in this tutorial will help you in the analysis of nearly any kind of FACS data.

The tutorial is only an introduction! FlowJo is capable of much that simply can't be covered in an introduction such as this (for example, there are Analysis Platforms to perform sophisticated DNA/Cell Cycle analysis, Kinetics analysis, software compensation, exporting raw gated data for analysis by other programs, etc.). You can learn more about FlowJo, and in particular how to use these platforms, through the on-line help facility. Whenever you ask for help from FlowJo, it launches a web browser (such as Netscape) and accesses help pages about the topic you selected. You can navigate the help pages to find out more

about all aspects of FlowJo. To access this on-line user manual directly, point your browser to "http://www.flowjo.com/v3/reference".

The online help documentation provides tutorials for Compensation, Kinetics, and DNA/Cell Cycle analysis; in addition, Tree Star provides Demonstration Data to let you explore these platforms.

In addition, there is a web page for FlowJo FAQs (Frequently-Asked Questions). Here you may find the answers to a problem you have had. FAQs are also instructive; you may learn new techniques from reading them. You may also wish to subscribe to a FlowJo EMail discussion group where you can post questions, answers, or tips. For more information, direct your browser to **www.flowjo.com**

Finally, we are pleased to be able to frequently update FlowJo to provide new features & analysis capabilities. Therefore, it is possible that the graphics shown in this tutorial may not exactly match the windows that you see when you run the most recent version of FlowJo. You can always download the most recent version of FlowJo from the web site listed above.

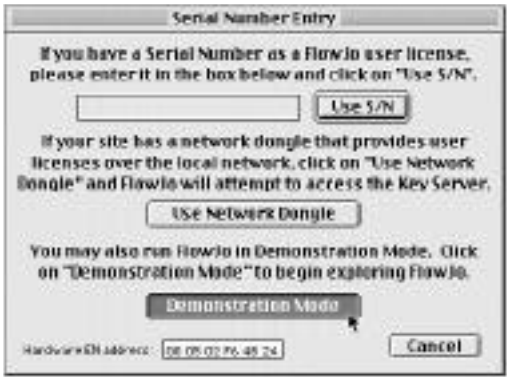
Table of Contents

Introduction 5
Lesson 1: Workspaces and Basic Data Display.....10
Lesson 2: Gating and Statistics 17
Lesson 3: Copying Analyses to Other Samples 26
Lesson 4: Groups and Batch Analyses.....32
Lesson 5: Modifying Group Analyses 40
Lesson 6: Tables and Layouts—Collating Data Output 50
Lesson 7: Creating Simple Graphical Layouts 57
Lesson 8: Creating Batch Graphical Reports.....72
Lesson 9: Generating Complex Batch Reports 81
Lesson 10: Creating Finished Reports.....90

Introduction

This chapter is designed only to give you a quick overview of the powerful batch features that FlowJo provides–don't worry about trying to learn how to use the program during this demonstration! Chapters 1 through 9 are designed to teach you details of using FlowJo. For this demonstration, you will load a sample data set into a previously designed Workspace that already has gates, statistics, and graphical reports designed. You can think of this workspace as a "Template," which could be used over and over for similar sets of experiments.

To begin this tutorial, first locate the "Tutorial" Folder on your Macintosh (either copied from the CD-ROM that came with FlowJo, or downloaded from the FlowJo web site). Open this folder; you will see a number of "Workspace" documents, and a folder containing the Tutorial data (it has within 2 folders of experimental data collected on different dates). Locate the workspace document named ***Demo Workspace*** and double-click it to launch FlowJo.


Serial Number Entry
If you have a Serial Number as a FlowJo user license, please enter it in the box below and click on "Use S/N".
Use S/N
If your site has a network dongle that provides user licenses over the local network, click on "Use Network Dongle" and FlowJo will attempt to access the Key Server.
Use Network Dongle
You may also run FlowJo in Demonstration Mode. Click on "Demonstration Mode" to begin exploring FlowJo.
Demonstration Mode
Cancel

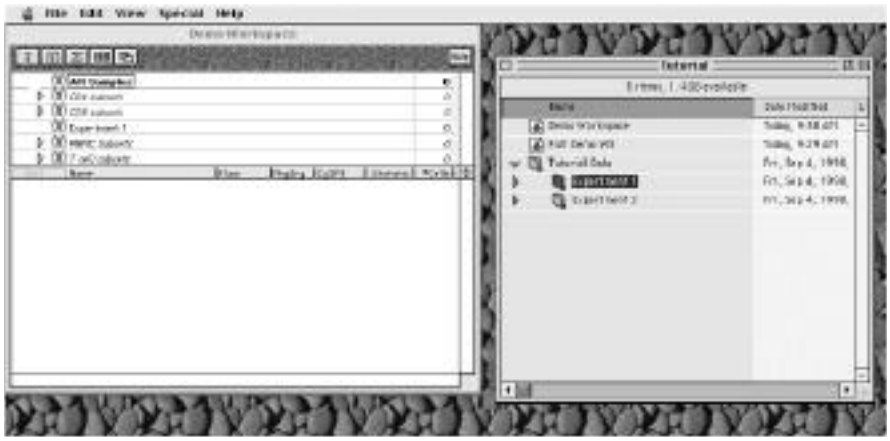
FlowJo is distributed over the Internet in a state where it can only read demonstration data. You can run this tutorial, or look at special FCS files that we provide, but it will not read your data until you have obtained a serial number from Tree Star. Serial numbers are machine-specific, so you need a serial number for every Macintosh on which you will run the program. We provide free evaluation serial numbers that let you run the program for a specific time period (generally sixty days). When you first run FlowJo, you will be presented with the dialog window shown on this page. (Once you enter a serial number, FlowJo won't ask for it again.) Note: The FlowJo application provided on the Demo Disk is already in "Demo" mode; you won't see this window. For now, you can click the Demonstration Mode button and continue.

Later, you'll want to request your own serial number to try out the program with your own data files. For more information about registering, point your web browser to ***www.flowjo.com,*** or send email to ***FlowJo@treestar.com.***

FlowJo opens the Demo Workspace and shows you the window at right: The organization of the ***Workspace*** window is more fully described in later chapters. For now, realize that the upper half of the window consists of ***Sample Groups*** which specify analyses (gates & statistics) to be applied to appropriately-stained samples, and the lower half of the window is the list of all samples in the currently-selected ***group*** ("All Samples" always includes every sample in the Workspace).

Because this is a template workspace, there are no samples in it.

There are several ways to load data into a workspace; perhaps the easiest is to drag a folder of data files from the Tutorial Folder in the Finder and drop it on the Workspace sample list. Click on "Experiment 1" in the



Finder (you may have to move the Tutorial Folder so that it is not underneath the workspace), and, without releasing the mouse, drag it over the lower half of the ***Workspace*** window. When you release the mouse button, FlowJo examines all of the files in the folder you dragged, and loads all FCS data files it finds. FlowJo can read data files collected on cytometers from any manufacturer. The workspace now reflects the new samples—and analyses (right). In this experiment, each sample was named by the date of collection (931115), a code related to the antibody panel (B,C, D, or E), and a sample identifier (e.g., "Sample 01").

Just by adding the data files to this workspace, FlowJo has already accomplished most of the analysis steps for this data! Each file was examined to see what reagent panel was used to stain the cells. Depending on the panel, FlowJo added the data file to one or more "Groups". Then, the gates, statistics, or other analyses, specified by each group were automatically added to the sample. Thus, gates and statistics specifically designed for each reagent panel were applied only to the sample tubes for which they were designed. You can scroll up and down the sample list to see the variety of different gates and statistics that were applied to each sample.

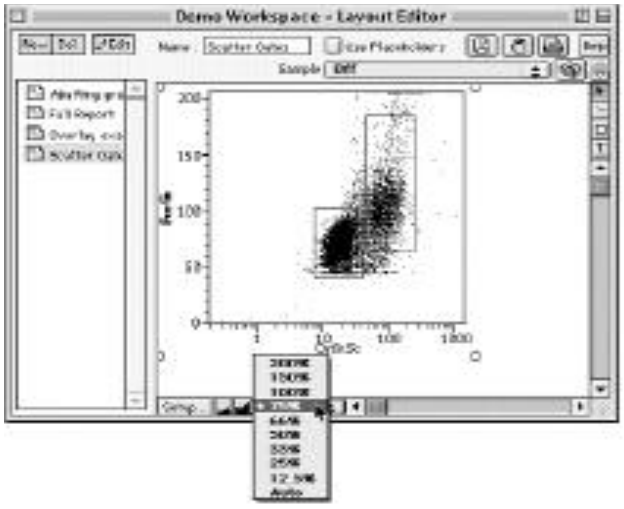
(Note that FlowJo has not yet computed the gates and statistics; you could request it to do so if you wanted right now. However, the default behavior is to compute them only when needed for the generation of tabular or graphical reports, as we will do momentarily).

This workspace also has several graphical layout reports saved with it. FlowJo saves not only analyses, gates, and statistics with workspaces,

but also any tabular and graphical reports, compensation matrices, kinetics & cell cycle analyses, and calibration standards. Everything you do is recorded and saved so that you don't have perform the same steps over and over!

To generate one of these graphical reports, you need to open the ***Layout Editor*** Window. You can either select this from the **Windows** menu, or click on the "Layout" icon in the ***Workspace*** window (as shown above).

FlowJo now shows you the ***Layout Editor*** window, opening one of the four graphical layouts that have been designed for this workspace (see graphic). In the left-hand portion of the ***Layout Editor*** window, you will see a list of different layout templates. Click on each one to see some examples of different layout formats. Finally, click on the Layout named "Scatter Gates".

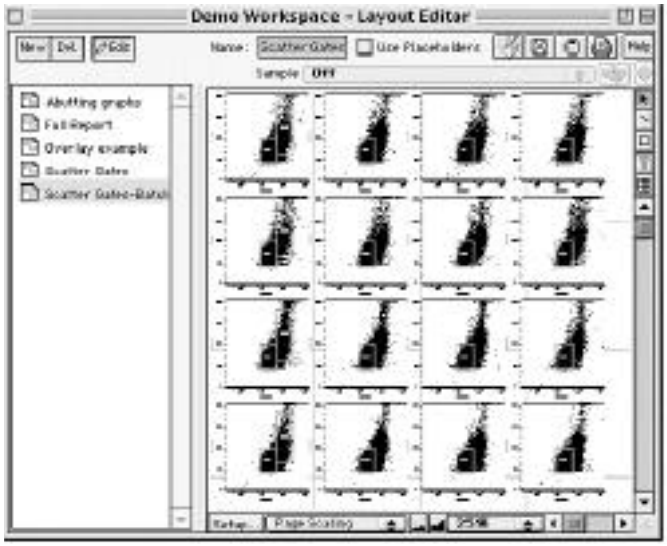


One of the important first steps in analyzing an experiment is to make sure that all of the scatter gates are appropriate for each sample. You will use this layout to verify the scatter gates for all 16 tubes collected in this experiment.

Change the magnification to 75% (as shown here). This gives you a better view of the Forward vs. Side scatter dot plot. If you wanted, you could now cycle through every plot by clicking successively on the "Next" or "Previous" sample arrows (just below the ***Help*** button). However, you can use FlowJo's Batch analysis to automate this process. Click on the "Batch Generation" Button (it looks like a stack of papers, right below the ***Help*** button).

From here you can choose from a variety of report formats. Leave the option set to New Layout to see a report like the one below. Or play around with the different options: the ***Tiled Report*** to see a matrix of all your plots, Web Report to view the output in a browser, or Movie to play each successive sample in frames of an animation. In Chapters 8 and 9, you will learn how to arrange the graphics in the Tiled Frames view to be printed in exactly the format you desire.

To demonstrate how fast this whole process can be, there is a second experiment's data also supplied with the Tutorial (in the folder Experiment 2). This is data for 14 more individuals (and is therefore much more extensive than Experiment 1). Drag this folder onto the Workspace (like you did for Experiment 1), and generate the batch outputs as before.



This example should convince you that with a well-designed ***Workspace***, you can completely analyze and generate custom graphics (and tabular) reports in a matter of just minutes for an entire experiment.

This is the true power of FlowJo: Experiment-Based Analysis.

Lesson 1: Workspaces and Basic Data Display

This lesson includes an introduction to FlowJo. You will learn how to start the program, along with the basic concepts of the Workspace, how to load data, and how to view some graphs.

FlowJo provides an integrated environment, the Workspace, for the viewing and analysis of flow cytometric data. The Workspace contains a list of all of the samples that you load into it, the gates that you apply, the statistics that you calculate, and the table templates and graphical layout templates that you design.

Think of the Workspace as your experimental notebook. In general, you will create a different Workspace for each kind of experiment that you perform. A Workspace may contain multiple samples collected on various days – and can provide a rigorous way to organize your data analyses.

To begin analyzing data in FlowJo, you will need to create a new Workspace and add data files into it. Later, you can simply double-click on this Workspace to continue work. For this tutorial, you will create a new Workspace and load a set of data files from a single collection. You will learn how to carry out basic analyses, including batch analyses. At the end of the tutorial, you will load a second experiment with a similar set of samples, collected on a different day. You will use all of the batch analysis tools you created in the first set of analyses and quickly derive detailed statistical and graphical information (in the forms of tables and printed graphical layouts) for both sets of samples.

As Workspaces are the basic environment that FlowJo uses to help you organize your data, they don't in fact contain the raw data. Instead, Workspaces have pointers to the data that you load. If you move the raw data files to another disk, then FlowJo will need to ask you to find the data the next time it needs that information. Workspaces do store much of the information about samples, as well as all of the analyses (gates and statistics) that you have previously computed.

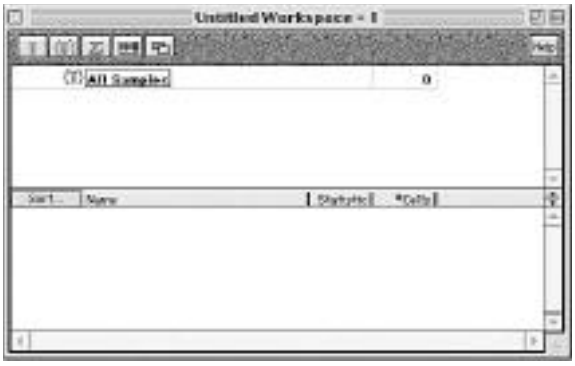
Introduction to the Sample Data Used in this Tutorial

There were two experiments, each performed on separate days. The experiments involved 3-color immunophenotyping of PBMC preparations from human adults. Each preparation was split into 4 aliquots to be stained with four different combinations of antibodies (see table); thus, there are a total of four tubes per PBMC preparation. (In general, we refer to each tube, or data collection, as a sample. In this experiment, there are four samples for each cell preparation.)

In this experiment there are four patients; in the second (folder "Experiment 2"), there are 14. The stain combinations used are as follows:

Stain #	FITC	PE	Cy5PE
1	CD14	CD16	CD45
2	CD3	CD8	CD4
7	CD62L	CD45RA	CD4
8	CD62L	CD45RA	CD8

As you work through this tutorial, the goal is to analyze the frequencies of some of the subsets that can be identified using these antibody stains, and to collate graphs and statistical information about subsets.



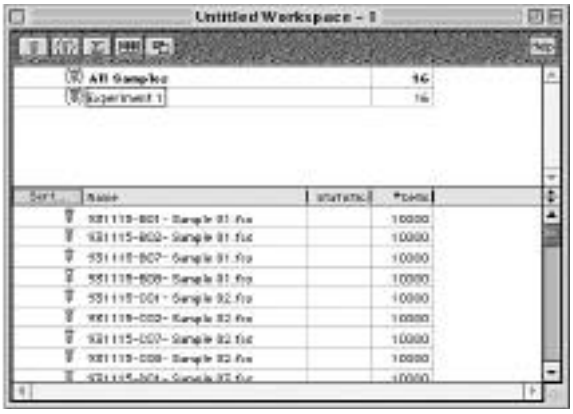
Once you have launched FlowJo, select **New** from the **File** menu. You will be shown a window similar to that shown to the right.

Note the ***Help*** button near the top right. Most windows in FlowJo have a ***Help*** button. When you click on ***Help***, FlowJo attempts to launch a web browser to get the on-line version of Help (or, you can

press the ***Help*** key on your keyboard at any time). The browser chosen can be set using InternetConfig; the default is Netscape Navigator. The help for FlowJo is HTML-based; when you ask for help, you will automatically be shown information about the active window. From the browser, you can navigate to all of the help pages for FlowJo, and learn how to operate the program. You can go directly to the main FlowJo help page by selecting **Help** under the Apple menu.

The ***Workspace*** window is divided into three parts. The top part is a button bar. The button bar has controls to let you add components to the Workspace, like data files, statistical analyses, etc. As you move the mouse over a control button, FlowJo displays a description of that control's action in the button bar. The second part of the Workspace (above the central divider) is a list of the current groups. Later, you will see how to group sets of samples together for batch analysis. The bottom part of the window includes a list of the samples in the Workspace.

Now, click on the left-most button, "Add DataFiles to Workspace". You will see a standard Macintosh Open File dialog; navigate to the "Sample FCS Data" folder that you downloaded with the tutorial (see above).



Highlight the folder "Experiment 1", and click on the button at the bottom to add all of the files in this folder (16 samples, one for each staining tube: four sets of PBMC, with four stains each). Your ***Workspace*** window should look as shown.

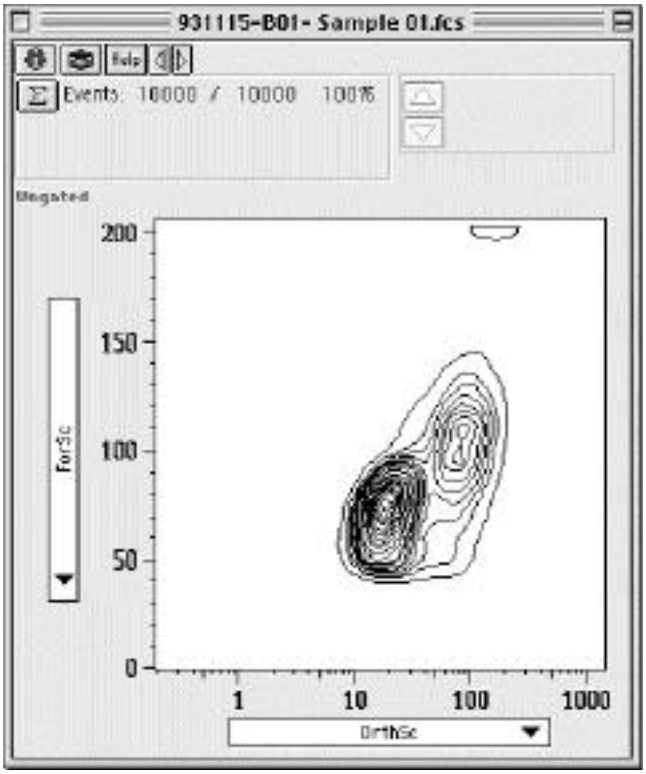
Two things have happened: FlowJo created a new sample group with the same name as the folder containing the FCS files ("Experiment 1"), and the samples are now listed in the bottom portion of the window. (The numbers to the right of each group show how many data files are included in that group.) You can resize the ***Workspace*** window as with any Macintosh window; you can resize the group and sample portions by clicking and dragging on the dividing segment.

Each file is shown as a test tube icon followed by the title of the sample's file. To the right is shown the number of events that were collected in that file.

You can add additional columns to the Workspace display that show the stains used for each sample, or any other keyword found in the FCS datafile. You may also sort the sample list on the basis of any of these values. To add the reagent list to the workspace view, click on any sample and select the command **Edit Workspace Columns...** from the **Workspace** menu. FlowJo shows the window above. Move the desired column headers from the list of available attributes (on the left) to the list of visible attributes (on the right). Click **Done** to accept the changes, and the Workspace view will add the additional columns to the table:

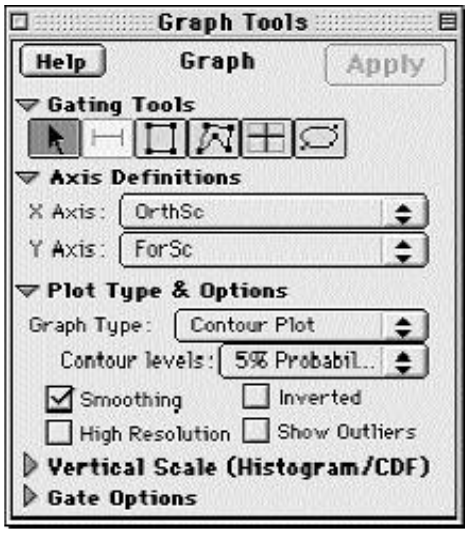
To view the data for any sample, double-click on one of the

lines in the sample list. When you double-click on the first line in the sample list, you will see the graph shown (right).



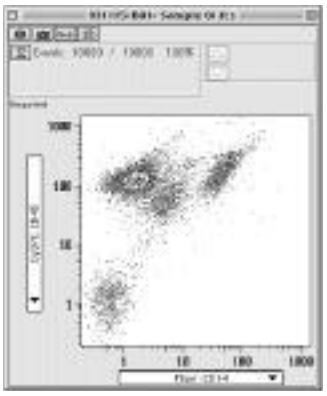
This is a ***Graph*** Window. A ***Graph*** window will show you a plot of the data.There are several different kinds of plots that can be used to display the data. Thedefault plot that you see the first time you open a file is determined by the Preferences setting. Select a different default plot by going to the **Preferences** dialog (under the **Edit** menu). Note that this selection only applies to workspaces you create subsequently; it doesn't affect the currently-open graphs.

The easiest way to change the graph is to click on the information button (with the encircled "i") at the top left of the ***Graph*** window. This button brings up the ***Graph Tools*** shown to the right.



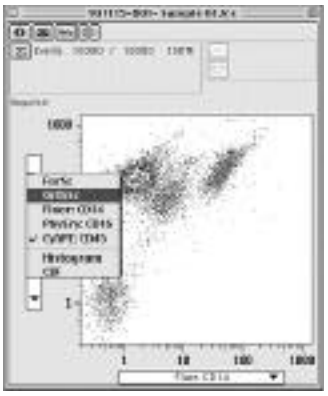
Here you can select the parameters you wish to view on each axis, the kind of graph to plot, and some of the options that affect the presentation of

the graph. Change the X axis to “CD14”, the Y axis to “CD45”, and the plot type to “Pseudocolor”.



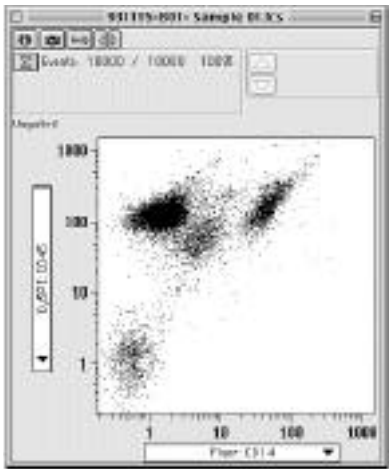
Click the ***Apply*** button in the top right corner of the window to activate the changes.

The data graph will now look like the one to the right. Another way to change the parameters on the graph is to click on the axis name: a list of parameters is shown in a popup menu. For example, to change the Y axis to show Sidescatter, click on the Y axis name box, and select “OrthSc” (Orthogonal scatter).

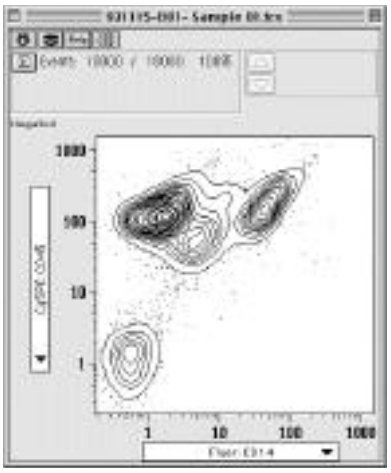


You may also choose “Histogram” or “CDF” (cumulative distribution function) to create a univariate plot. Experiment with different graph types and different options. You can change individual options by selecting them in the **Graph Menu.** (Try deselecting **High Resolution** for the pseudocolor plot; this type of plot – low resolution pseudocolor – may be the best for slide presentations.) In general, the probability contour plots give the best representation of the data, though they are lacking in that they do not show rare events. To work around this, FlowJo provides the option of displaying “Outliers.” Outlier plots combine the density estimation information of the contours with the rare event information of a dot plot. The density plots (either grayscale or pseudocolor) are superior to dot plots in that they provide density information (i.e., the number of events within an area) by using different colors or shades. Grayscale plots may be useful for publication and pseudo-color plots for slide presentations. Shown on the next page are examples of some of the other types of plots supported by FlowJo.

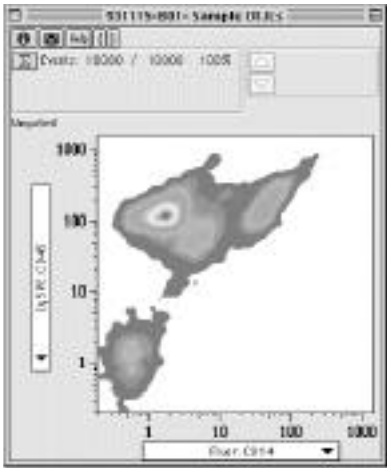
When you close a ***Graph*** window, FlowJo records the plot style and axes. When you next open the graph for that subset, you will see the graph as it was when you closed it. This is, in part, how FlowJo saves the environment across analysis sessions.



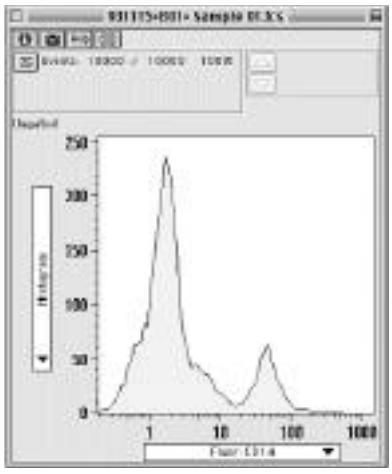
Dot plot



Contour plot with outliers



Smoothed pseudocolor plot

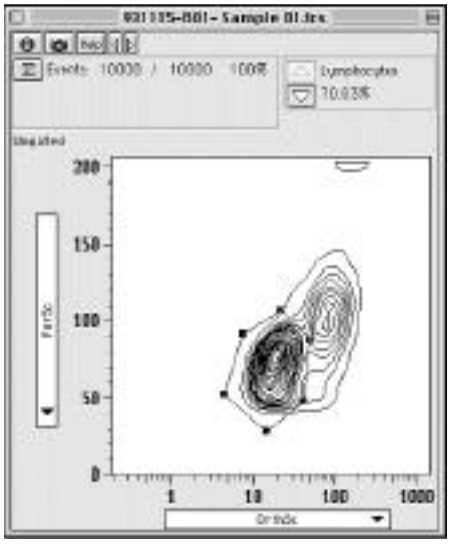


Histogram plot

Lesson 2: Gating and Statistics

In this lesson, you will learn how to "gate" on data to create subsets. You will also learn how to calculate a variety of statistics from the data. Subsets in FlowJo are exactly like subsets in biology: they represent a subset of the entire collection with specified properties (e.g., "Lymphocytes" are those cells with low Forward and Side Scatter). You will always be asked to name a subset; the name you choose is important for organization within FlowJo. Select a name that is meaningful to you, as this will help you keep track of your analyses.

Creating a gate is simple: just click inside a graph (when the mouse appears as a cross-hair) and move the mouse. Each time you want to change directions, click and continue to drag. This is the same process as you would use to draw a polygon in any drawing program. If you hold down the ***shift*** key, you get horizontal or vertical lines; you can use the ***delete*** key to remove the last vertex. Either click on the first point, double-click at any time, or press any key (other than ***delete***) to close the polygon and create the gate. Hold down the ***option*** key to make a rectangular gate.



Open the first file in the sample Workspace you created and change the graph to Forward vs. Side-scatter with a contour plot. You will now create a lymphocyte gate: draw a new gate around the lower population, as shown above.

As soon as you close the polygon, FlowJo asks you to name the subset that you have just gated (see next page). FlowJo provides a default name; *however, you should choose a name that describes the population that you gated.* Names are very important within FlowJo analyses. The

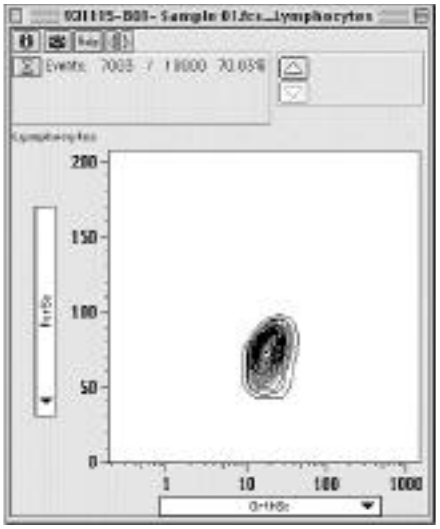
success of your future analyses depends on having analyses with descriptive names. For example, in this case you have gated lymphocytes, so name this subset "Lymphocytes". Type the name into the box. (The Help button will provide more information about naming subsets.) If you click on the Select button, FlowJo then creates the gate, and opens a new ***Graph*** window showing only the events contained within the gate you just drew. For now, just click on the **OK** button.

You will note some changes to the ***Graph*** window. The box in the upper right has the name of the currently-selected gate and the fraction of events in the entire sample that fall into the subset. In addition, the Down-arrow button is now active. If you click on the Down-arrow, FlowJo opens a new graph showing the data contained only within the "Lymphocytes" gate.

Also, there is a new entry in the ***Workspace*** window (see example, left).

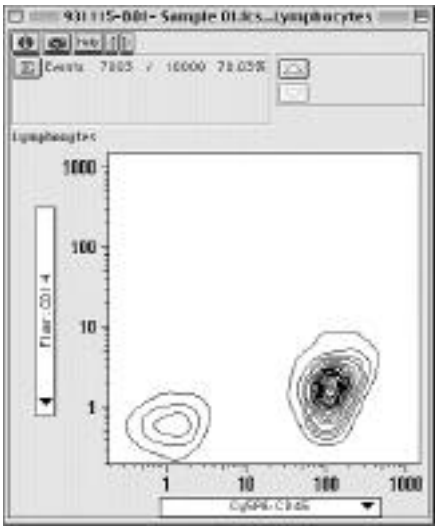
Underneath the sample you just gated, indented one level, is a new row. This new row represents the subpopulation defined by the gate. This row begins with a subset icon, followed by the name that you gave to the subset. To the right, you will find the frequency of these events (within the sample) and the total number of events in this gate. Anything that can be done to a sample, can also be done to a subpopulation. You can double-click on the "Lymphocytes"

subset to open its graph, gate within that subset, etc. Double-click on this population. You will see the graph to the right.



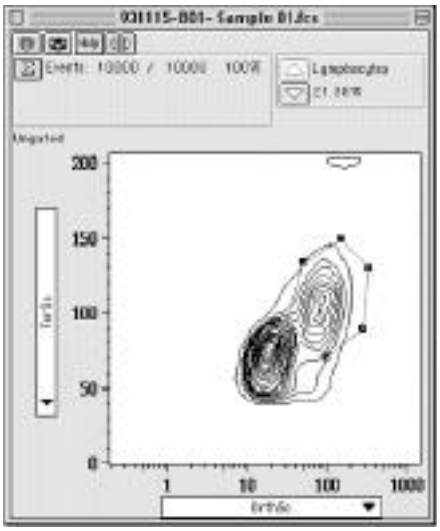
This is the scatter distribution for the events falling within the lymphocyte gate. Note that the number of events in this graph (top left) is 7003, which is the number of cells falling in the lymphocyte gate. You will also see that the Up-arrow button is now active. Clicking on this button opens the graph you used to create this subset (i.e., you will see the gate for this subset). The Up-arrow can be used to navigate to the parent population; the Down-arrow navigates to the subset. Click on the down-arrow (or double-click on the gate in the graph) to show a new ***Graph*** window for the subset (see below).

This graph is not particularly interesting, since it shows a subset of the same data as the previous graph. Change this graph to CD14 vs. CD45; your graph now will look like the one to the right. Note that most of the events are CD45-positive and CD14-negative: this is exactly what we expect for lymphocytes in PBMC. The CD45-negative population is probably red blood cells that contaminated the cell preparation. Move this window to the side, and click on the Up-arrow button to bring the parent population visible. You should be able to see both graphs simultaneously.



FlowJo uses dynamic recalculation of gates. This means that whenever you

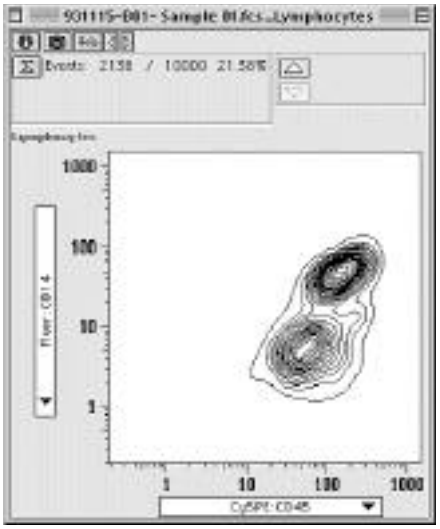
adjust a gate, FlowJo automatically updates all visible windows to reflect that change. Move the mouse over the lymphocyte gate in the ***Graph*** window; the cursor changes to a hand. If you now click and drag, you will move the gate. Move the gate so that it is over the monocyte population as shown (left).

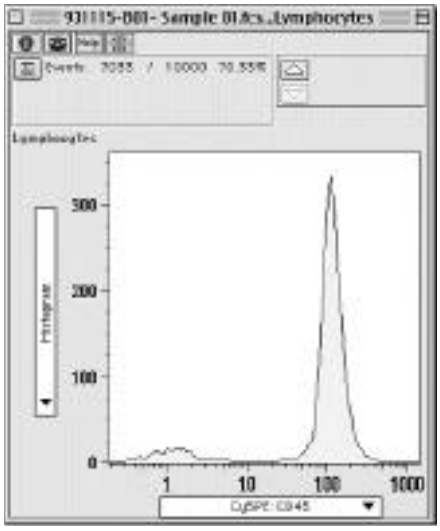


FlowJo instantly updates the graph of the subset; that window will now look like the one below right.

The new gate includes events that are all CD45-positive, and a large fraction are also CD14-positive (i.e., monocytes). You can continue to move the gate around, and see exactly how the gating affects the subpopulations. These changes also occur when you make minor modifications to a gate (like clicking and dragging a single vertex).

Move the gate back to the lymphocyte population so that the gate accurately reflects lymphocyte cells. Change the graph for the lymphocyte population – click on the Down-arrow to get to the Lymphocyte ***Graph*** window, or simply select the corresponding ***Graph*** window – so that it displays a histogram of CD45. Select "Histogram" using the Y axis pop-up (or use the ***Graph Tools*** palette to define the graph type).



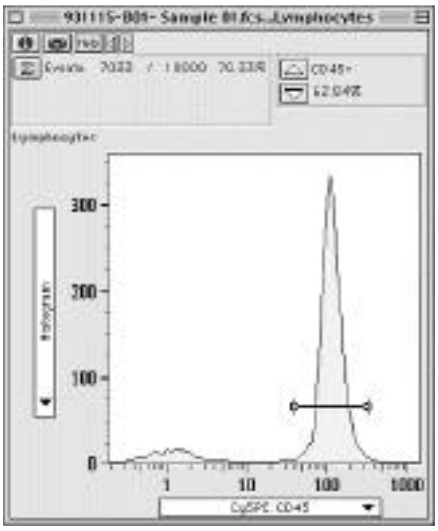


The graph should now appear as shown on the left.

If you wish, you can change the vertical scaling on univariate plots through the controls in the ***Graph Tools*** palette.

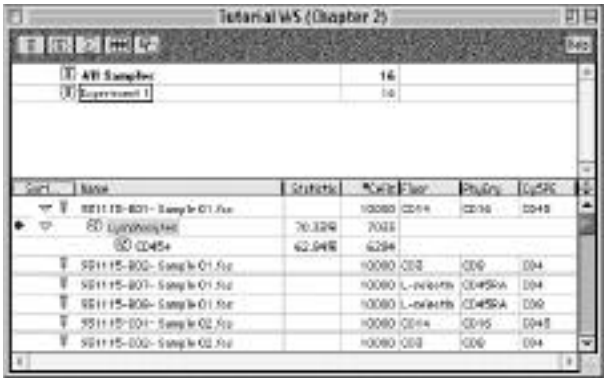
Creating a gate on a histogram is the same as creating a gate on a bivariate plot: click and drag in either direction. Make a gate to include only the CD45-positive cells. Click on one side of the peak and drag to the other side. When you let go of the mouse, FlowJo again asks you the name for this new subpopulation (subset of cells). Type in "CD45+" as the name of the subset.

The ***Graph*** window now displays the gate, and the statistics for that gate, as shown below. The vertical placement of the gate is irrelevant; you can drag the gate horizontally or vertically by clicking on the blue bar (the cursor will be a hand) and moving it around. You can change the upper or lower boundaries by clicking on one of the handles and moving it.



The ***Workspace*** window now has a new entry, as shown in the example on the next page.

Note that the new entry is below Lymphocytes and is further indented. This is because the new subset is a subset of Lymphocytes. The Workspace reflects this hierarchy. The numbers to the

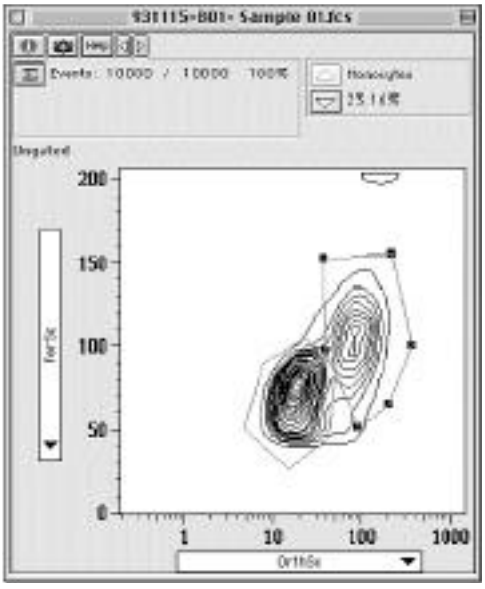


right indicate the fraction of events falling within the gates. Thus, 70.5% of the sample's events fall in the lymphocyte gate; 63% of the events fall within both the lymphocyte gate *and* the CD45+ gate.

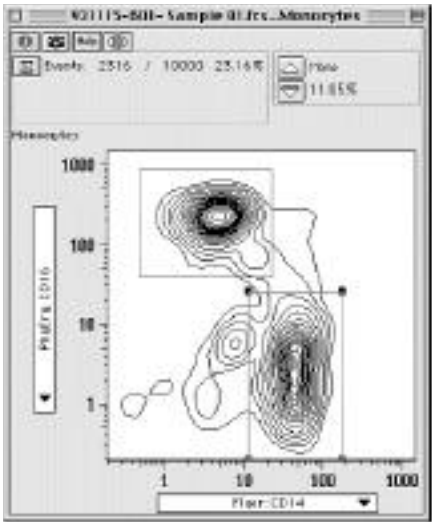
At this time, we will add some statistics to one of these subsets. Click on the "CD45+" row so that it is highlighted, and then click the middle button in the button bar (the Sigma button, ∑). This indicates that you want to calculate a statistic of the currently-selected subset. You will now see the ***Statistics*** window, as shown to the right.

On the left side of of the ***Statistics*** window is a list of the statistics available. Some of these statistics require that you choose a parameter on which to calculate the statistic (for example, "Mean fluorescence"). If you wish to compute a specific percentile of one of the parameters, you will type the percentile into the numerical box. For now, click on **Freq. of Parent**, and then click on **Add.** Also, add the **Freq. of Grandparent**, and add the **Median** and **CV** for Cy5PE CD45 (select the parameter from the **Channel** popup menu). If you click on **Help**, a corresponding web page will be opened with your web browser that gives you complete information about the various statistics. When you have finished adding statistics to this population, close the window. Once you close the ***Statistics*** window, your ***Workspace*** window has several new entries as shown on the next page.

The new lines begin with a statistics icon. (You can click in the middle column name bar on vertical dividers and drag to resize columns, to show the entire names). The statistics icon is followed by the parameter on which the statistic is computed (if applicable), the name of the statistic, and the computed value. The median Cy5PE CD45 fluorescence of the CD45+ Lymphocytes is 114; the distribution of CD45 has a coefficient of variation of 33%. The **Freq. of Parent** statistic tells you what the frequency of CD45+ events is, within the parent subset (Lymphocytes). You will note that the value, 89.8%, is equal to 63% (the frequency of this subset within the sample) divided by 70.5% (the frequency of lymphocytes within the sample). The **Freq. of Grandparent** shows you the frequency of this subset within the subset two levels up – in this case, the sample itself. Thus, for this subset, the Freq. of Grandparent is the same as the frequency of this subset within the sample. These statistics are listed below the "CD45+" subset to denote that they are statistics for that subset only.



Next, you will create another gate on the sample, this time for monocytes. Open the graph for the entire sample, choosing the Forward vs. Side-scatter display option. Draw a gate around the larger population as shown to the left, and name this subset "Monocytes."



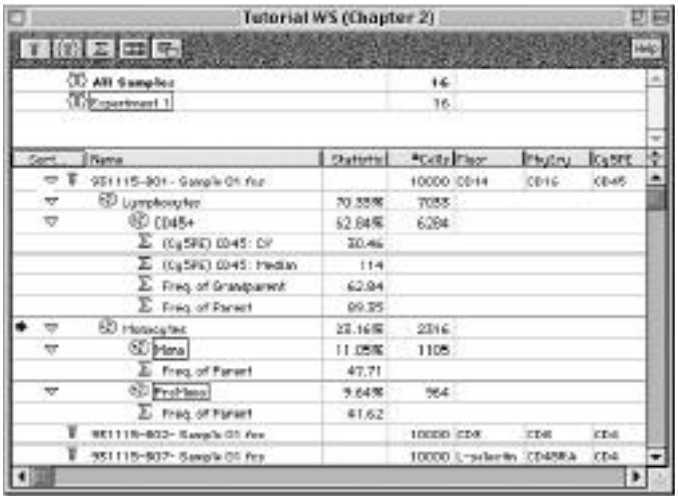
Note that the currently-selected gate is the one with the handles drawn at each vertex. The frequency of events in the currently-selected gate (within the entire sample) is shown in the upper-right portion of the window; when you click on the Down-arrow (or double click on the gate), the ***Graph*** window for the subset will be shown. Click on the Down-arrow to view the monocyte subpopulation. Change the resulting graph to look at CD16 vs. CD14.

Create two gates for the major populations that you see. This time, press the ***option*** key while you click for the first time to create the gate – FlowJo will restrict your gate to be rectangular. Rectangular gates will be computed much more quickly, but this is only evident when you work with very large files (for instance, more than 100,000 events). You may find rectangular gates easier to create. Create both gates to be rectangular; see the graph above.

Name the upper gate (CD16+, CD14-dim) "ProMono" (although this may well be granulocytes; you could name it "Granulocyte" if you wish). The lower right population is principally mature monocytes; name it Mono. Look at the Workspace window. You will see the new subsets displayed; click on the ProMono subset and add the Freq. of Parent statistic; then do the same for the Mono subset. The Workspace now looks like the Workspace shown on the next page.

Note that the Monocyte subset is indented one level, as is the Lymphocyte subset. Both populations are subsets of the sample. The Mono and ProMono subsets are indented to a second level and are shown below Monocytes, because they are subsets of monocytes, not of lymphocytes. This hierarchy of this gating is visible from the ***Workspace*** window.

This hierarchy is important in that if affects how FlowJo works. It is analogous to a genealogical tree, in that each subset is like a "child" of its parent population. Thus, in this example, "Lymphocytes" is a "child" of the sample, and a "Parent" of "CD45+", and a sibling of "Monocytes."



FlowJo does not allow you to name "siblings" (i.e., gates on the same population) with the same name – this way no confusion can arise. You can, however, give the same name to gates under different subpopulations. Later, you will see examples of how this is useful to more complex analyses.

FlowJo identifies subsets using the names of populations. When you later create graphical layouts or tables, you tell FlowJo that you want information about a population (like CD45+ Lymphocytes). FlowJo will look within all of your samples for a subset or statistic with this name (and ancestry) to extract the information you want. Importantly, the precise Lymphocyte or CD45+ gate can be different between different samples, but FlowJo will still recognize the populations and get the data you want from them. In fact, you can even draw a gate on completely different parameters – but if you name it "Lymphocytes" and it is at the sample level, then FlowJo assumes you have selected the same *kind of cells* as every other "Lymphocytes" gate attached at the sample level.

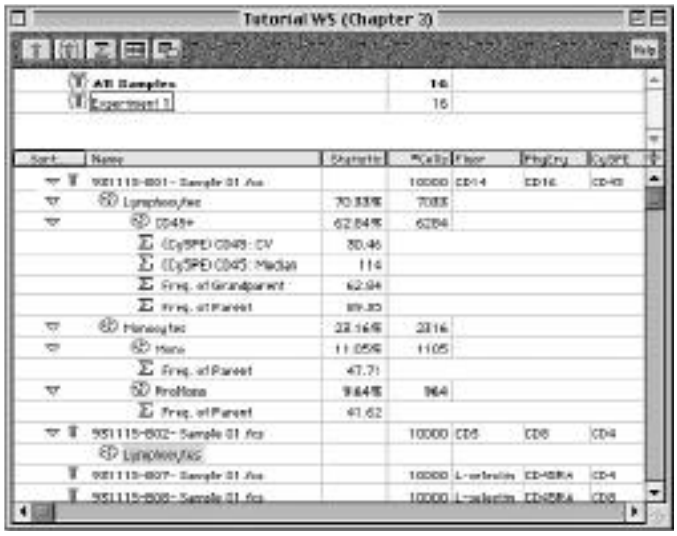
In conclusion: The name of a subpopulation is the fundamental name by which FlowJo identifies subsets of cells.

Lesson 3: Copying Analyses to Other Samples

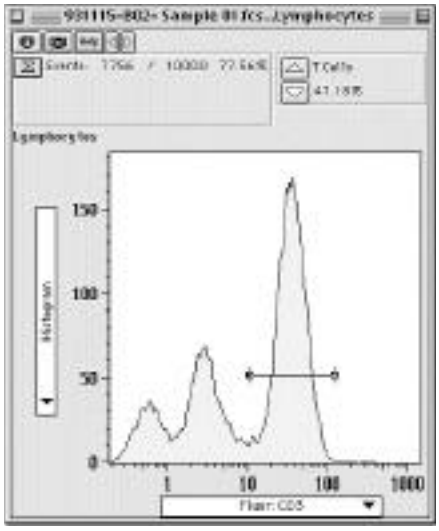
One of the basic principles of FlowJo is that virtually all analyses that you perform on a population of cells (a sample or a subset) can be applied to other populations using the "drag-and-drop" feature. Hence, when you click on an analysis (for instance, a gating to create a subset or a statistic), and you drag it to another place in the Workspace, FlowJo copies the analysis into the new location.

Now, it is time to analyze another set of cells from this same patient. This lesson builds on the Workspace you finished in Chapter 2; alternatively, you can open the workspace named "Tutorial WS (Chapter 3)". We will analyze the second file in the Workspace, which has the sample from patient one stained with the second combination of reagents.

Here is where you will get the first glimpse of the powerful batch analyses that FlowJo can perform. You have already made a lymphocyte gate on stain 1; presumably, this same gate should be applied to stain 2. To do this, simply click on the Lymphocyte subset in the ***Workspace*** window, and drag it down so that the second sample row is highlighted; then, let go of the mouse. FlowJo creates a new subpopulation, using the same gate you had previously created. Your ***Workspace*** window now looks like the one shown here:



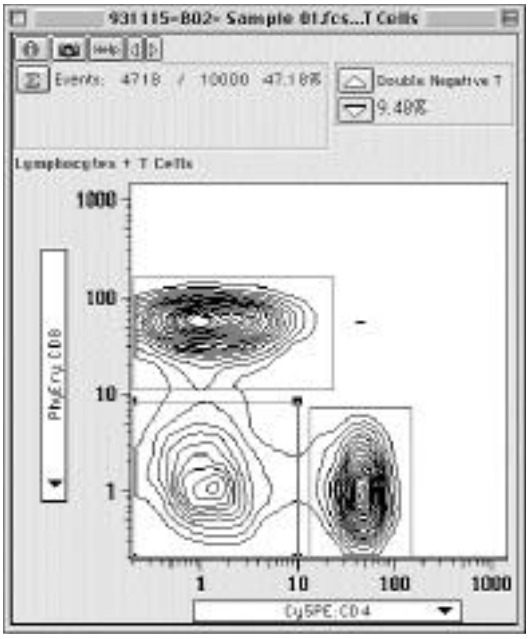
Note that there are no numbers to the right of the new Lymphocyte subpopulation (depending on the current setting of your Preferences). FlowJo does not, in



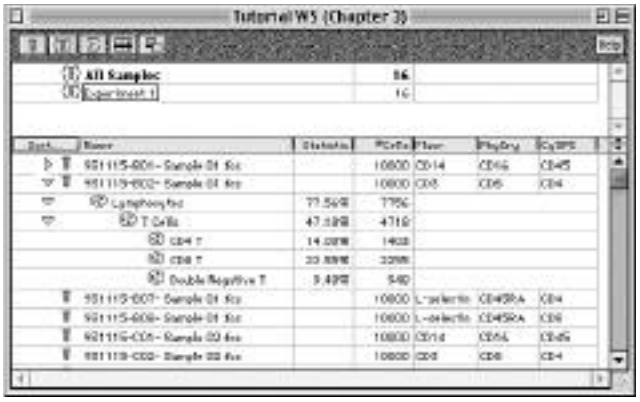
general, calculate statistics (such as frequency) until they are absolutely needed – the display in the Workspace is not considered a high priority. (Later we will see how to override this behavior.) In any case, you can still double-click on this population to open a graph of the lymphocytes within the "931115-B02-Sample 01" file.

Now, open this graph, and choose to view a histogram of CD3. Create a gate on CD3+ cells, and name it "T cells." At this point, your ***Graph*** window should look like the example here (top left).

Click on the Down-arrow to open a graph of the T cell subset. Create three rectangular gates on the CD4 single-positives, CD8 single-positives, and the CD4-negative, CD8-negative (double-negative) T cells, as shown to the right.

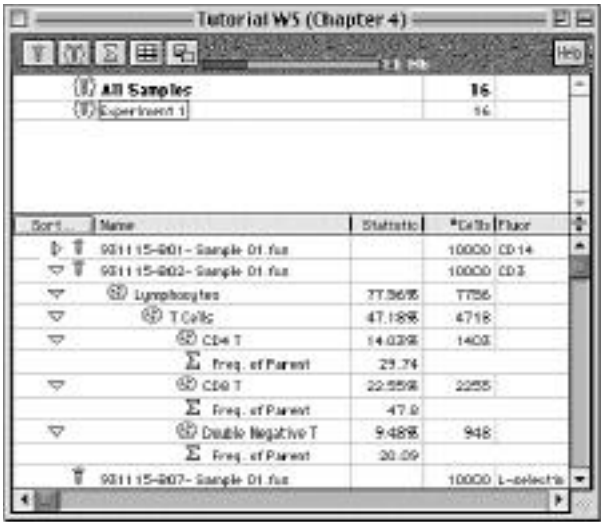


In the ***Workspace*** window, click on the triangle next to the very first line. These triangles open and close the views of the subset hierarchy, much like they do in the Macintosh Finder. In the genealogical terminology that FlowJo uses, the "CD4 T", "CD8 T", and



"Double Negative T" subpopulations are siblings, and are children of the "T cells" subpopulation, grand-children of the "Lymphocytes" subpopulation, etc. Remember that the frequencies and cell counts shown to the right are with respect to the entire (ungated) sample. The ***Workspace*** window (above) reflects this hierarchy.

To calculate the relative representation of these three subsets within T cells, add the Freq. of Parent statistic to each subset. You may also add it to the first subset (CD4 T) using the "Add Statistic" button, and then just click and drag the new row to the other subsets to save time (i.e., you are copying analyses from one population to another). Note that dragging a statistic from one population to another is a way of copying the mathematical operation. See the example below. You could also select all three subsets (***shift***-click them), and add the statistic to all at once from the ***Statistics*** window.



Click on the triangle next to the "Lymphocytes" gate to collapse the view. A second patient sample stained with the same reagents is found a few rows down, labeled "931115-C02- Sample 02." Again, we would like to apply exactly the same gates and statistics performed on the first patient

sample to the second. We could drag each line one by one to the second sample. However, FlowJo provides a special mechanism for copying entire analysis trees: if you hold down the ***option*** key as you begin to drag, then FlowJo will take the subpopulation you clicked, as well as all of its descendants. You will see this occurring via the dotted outline that FlowJo draws, which denotes all of the analyses that you are copying.

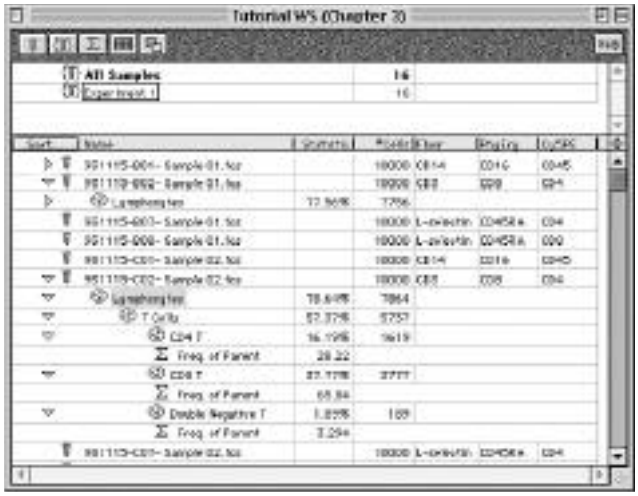
Click on the Lymphocyte gate, press the ***option*** key, and drag it to the "C02" row. (Even though the children of the first lymphocyte gate are hidden because you closed the triangle, they are still copied). Your workspace will now reflect the fact that you copied 8 different analyses (gates and statistics) with one operation.

There are more complex ways of selecting which analyses to copy. For instance, you can ***shift***-click several analyses to drag multiple different gates simultaneously, and/or you can use the ***control*** key to ask FlowJo to take the ancestors of the subset you are selecting. These actions can be combined. For a detailed description of these operations, go to the on-line help documentation, and find the section about dragging and dropping analyses.

Again, you will see that FlowJo has not actually calculated any of the analyses you requested. If you wanted to see the results right now, you can force this calculation to occur. Click on any population, and select **Recalculate...** from the **Workspace** menu. (Note, you can also select a Preference that will control whether these recalculations should always be performed immediately).

The Recalculate command tells FlowJo to calculate the gates for the currently-selected node, and to perform all statistical and gating calculations for any of its children. Select the new lymphocyte subset, and select **Recalculate** from the **Workspace** menu or press ⌘-=. FlowJo reads

in the data file, and makes the necessary computations. The workspace now appears as shown.



You can delete analyses by selecting them and pressing the ***delete*** key. Select the lymphocyte subpopulation that you just created, and press the ***delete*** key. FlowJo asks you to confirm your choice, telling you what the consequences will be (below). Note that there is a help button; clicking on this will take your web browser to help specifically about deleting nodes ("nodes" are analysis rows in the ***Workspace*** window).

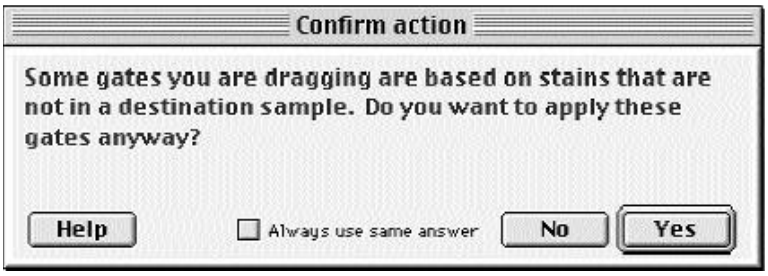
Confirm action
Deleting nodes also deletes all analyses belonging to these subsets (children). Are you sure you want to delete the selected node(s)?
Help
Always use same answer
No
Yes

When you delete the lymphocyte gate, all of the subsets of lymphocytes will also be deleted– this is because those subsets have no meaning without a lymphocyte gate being present. Remember, every subset that you name is, in reality, a subset defined by all of the gates of its "ancestors"-the parent gate, grandparent gate, etc. When you delete a gate, FlowJo lets you know that all of its descendants will also be deleted in the confirmation request.

One more detail: when you create a subset, the gate used to create that subpopulation remembers both the parameters *and* the stains on which the gate was drawn. In other words, the CD4 and CD8 T cell gates

drawn above were created on a sample that had PE CD8 and Cy5PE CD4. If you were to drag this population to a sample where you did not collect a Cy5PE channel, then FlowJo could not copy the gate and nothing would happen. However, if you copy to a sample that has both PE and Cy5PE, but the stains are different, then FlowJo displays a warning.

For example, drag the CD4 T gate from sample C02 and drag it to sample C01. FlowJo presents you with another confirmation window, shown below.


Confirm action
Some gates you are dragging are based on stains that are not in a destination sample. Do you want to apply these gates anyway?
Help
Always use same answer
No
Yes

FlowJo is warning you that the CD4 gate was drawn on PE CD8 vs. Cy5PE CD4, and may not be applicable to the sample stained with PE CD16 and Cy5PE CD45. (In fact, in this case, the gate is not appropriate.) You can, if you wish, choose to copy it anyway, and then the gate would be applied to the PE and Cy5PE channels irrespective of the stain.

For this (and other) reasons, it is important that you carefully enter the appropriate stain names when you collect the samples. Naming stains appropriately is good practice anyway – you will then always have properly annotated data!

Of course, another good practice is to save your workspace files. The workspace does not have to be saved in the same location (or even same disk) as the data files; the workspace remembers where the data files are. Note that there is an option in the Preferences to have FlowJo periodically ask you to save the workspace–and, by clicking in the "Use Same Answer" checkbox the first time FlowJo asks you to save the workspace, it will then automatically save the workspace every few minutes!

Lesson 4: Groups and Batch Analyses

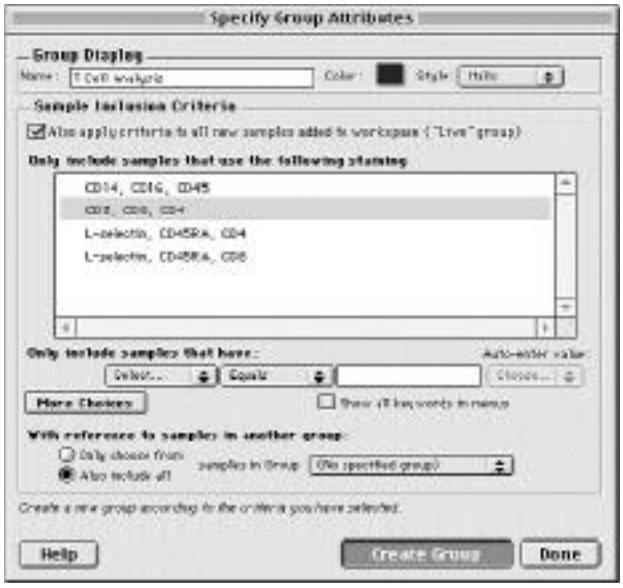
In this lesson, you will learn how to take advantage of sample groups. Groups are the primary mechanism by which FlowJo allows you to perform batch analysis. You may create as many groups as you wish; each group can consist of any of the samples within the Workspace (and samples may belong to more than one group). In general, doing something to a group will be equivalent to doing the same thing to every sample in that group—the advantage is that you do it all at once.

In Lesson 3, you learned that by dragging multiple nodes, or entire trees of nodes, you can accomplish the first step in batch analysis: the ability to replicate multiple analyses simply, and with the guarantee that the copies are identical to the originals. But that still leaves the next step: how can these analyses be applied to a whole set of samples at once? To accomplish this, FlowJo allows you to create groups of samples. A group, which is simply a list of samples from the Workspace, can then serve as a sample surrogate. That is, you can apply analyses to a group much as you can apply them to single samples.

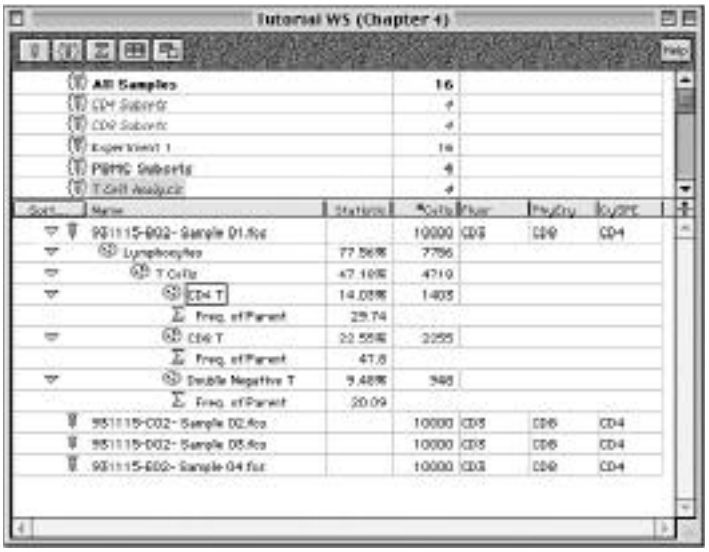
A group may consist of any number of samples from the Workspace. Any given sample can belong to as many groups as desired. However, there is one rule about groups and group analyses that is inviolate: every sample in a group has every gate, statistic, or other analysis specified by that group (as long as it is applicable to the sample). This will become more clear in the next few steps of the tutorial. This lesson builds on the Workspace you finished in Chapter 3. Or, open our premade workspace named “Tutorial WS (Chapter 4)”.

The first step is to create a group that contains a set of samples which will receive similar kinds of analyses. In our case, we will begin by analyzing the samples stained with the second combination of antibodies (CD3, CD4, and CD8). In the Workspace, click on the second button in the button bar, “Create New Sample Group”. FlowJo brings up the window shown on the next page. Using this window, there are a variety of ways to make new groups. In the middle portion of the window is

listed every different combination of stains present in the whole list of samples. They are presented in channel order (in this case, “FITC, PE, Cy5PE”). Stains which were left blank at the time of collection would be noted with an ellipsis (…). Click on the CD3, CD8, CD4 combination, telling FlowJo to select all samples with this combination. Enter the name “T Cell Analysis” in the box at the top, and click on the Create button.



The window is still present (you will notice a new line in the ***Workspace*** window corresponding to the group you just created). You will now create your second group, corresponding to the analysis of the CD14, CD16, CD45 stains. Again, click on the stain combination, and enter the name for this group (“PBMC Subsets”). Before you create this group, change the color to red (click on the color box), and select Bold from the Style popup menu. Your window should look as shown to the left.



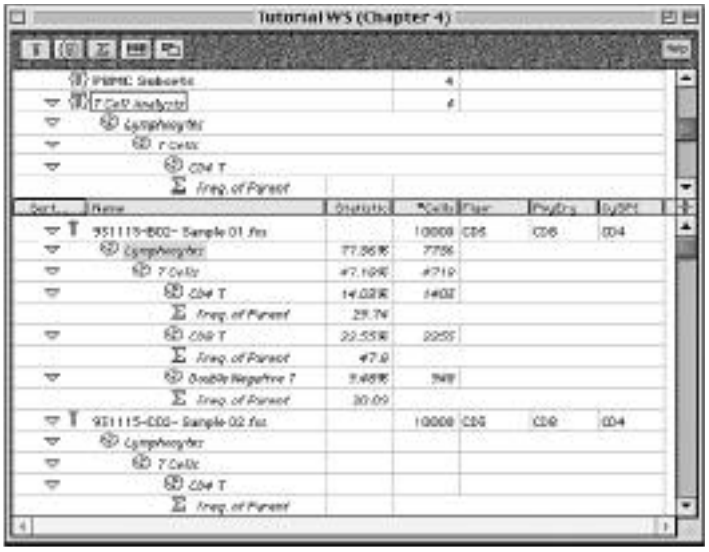
Now click the Create button to create this group. Finally, make two more groups, called “CD4 Subsets” and “CD8 Subsets”, adding the third and fourth stain combinations, respectively, and set the color to green and the style to italic. When you are finished, click Done. Your Workspace window, after you click on the “T Cell Analysis” group, should look similar to the one shown here.

Each group is displayed in the upper (group) list, in the color and style you have chosen. The number to the right of each group tells you how many samples are included in each group. Since there were four patient PBMC preparations, each stained with each combination of antibodies, each group has four samples listed. Click on each of the groups, and verify that a different set of four samples is present in each of the different groups. Finish by selecting the “T cell analysis” group.

Remember that a group is a sample surrogate. Thus, any analysis that you apply to the group is applied to all of the samples in that group. To see this in action, click on the “Lymphocytes” gate. Hold down the option key while dragging (so that the entire tree of analysis is copied), and release the mouse when it is above the “T cell analysis” group in the upper part of the window.

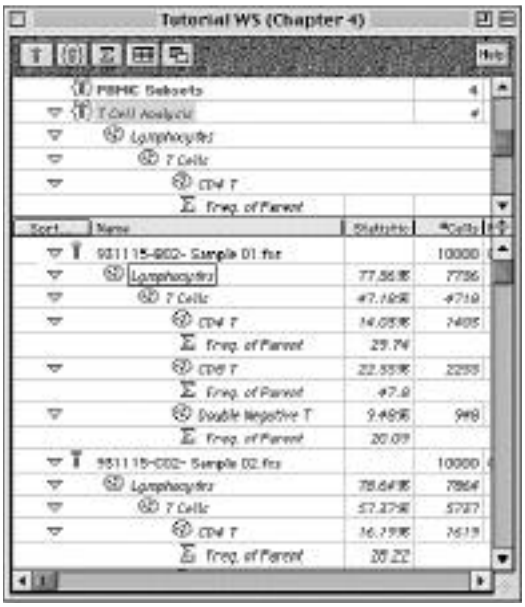
Notice that several things have happened. First, the analysis tree has been added to the group row (as if it were a sample). Second, the analy-

ses have been added to every sample in the sample list. Third, all of these analyses now appear in blue and italic, which is the same color and style that you defined for this group. You can see all of these in the example workspace on the right.



The color / style annotation of the analyses is an important cue for you to note. Any analysis that appears in the Workspace in the color and style of the group is guaranteed to be exactly the same as the group's version. Thus, the gate will be in exactly the same location, applied to the same parameters, etc. This is how you can guarantee that the exact same analyses have been done on all of your samples. (We will see later how to modify analyses for particular samples.)

If you scroll down the sample list, you will note that while the analyses (gates and statistics) were added to all of the samples, not all have been computed. Again, FlowJo only computes these when it is necessary (or if the sample is currently in memory). To force FlowJo to recalculate all of these at once, you will use the Recalculate tree... command. First, select the group node ("T cell analysis"), and make sure it is highlighted. Now choose the Recalculate Samples command from the



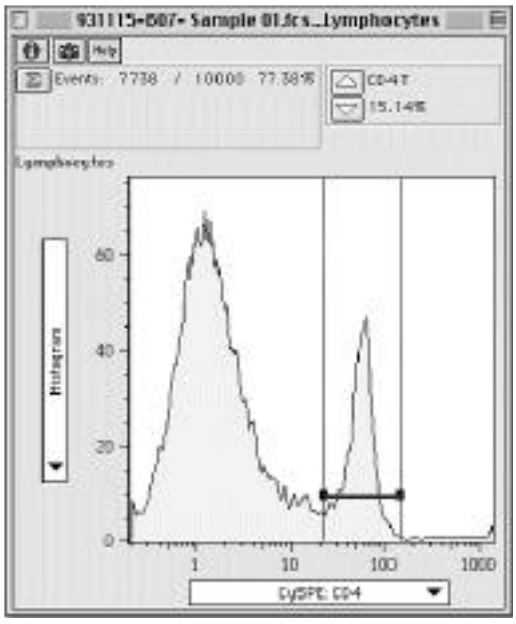
Workspace menu. Since the currently-highlighted row is a group, FlowJo applies the command to all of the samples in the group (i.e., the group serves as a substitute for each and every sample in its list). After you perform the **Recalculate...** command, you should see that all of the samples have been computed (see example, left). We will also use the command **Edit Workspace Columns...** to remove the reagents from the workspace to simplify our view. You can choose to display or hide whatever attributes you prefer.

Now, we move on to the next set of analyses, the CD4 and CD8 subsets. Again, we will use groups to speed up the process (and ensure that identical gates are used for common gatings, like lymphocytes). While the CD4 and CD8 subset analyses used different stains, we can still copy the lymphocyte gate, since it is based only on Forward and Side scatter. Click on the Lymphocyte gate from one of the samples (e.g., the first one) in the current group, and drop it on top of the CD4 subset group. The gate is

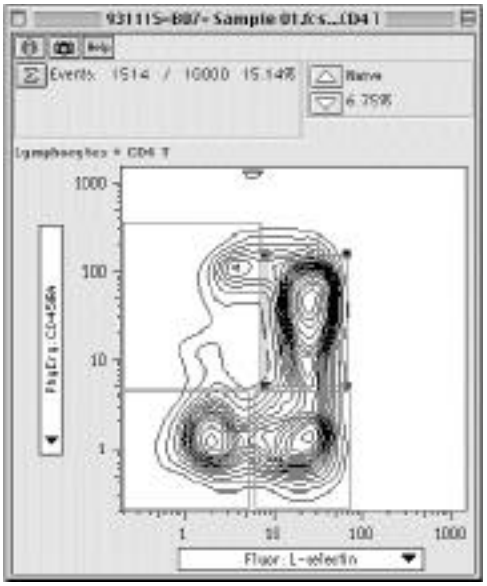
now applied to all of the CD4 subset samples.

When you click on the CD4 subset group, you will see the four samples with the lymphocyte gate (now drawn in green italic), as shown on the right.

Double-click on the first sample's lymphocyte subset, and change the graph to show a histogram of CD4. Create a gate for the CD4+ T cells like the one shown below.



Click on the Down-arrow in the box at the upper right corner of the Graph Window, in order to view the population defined by the active gate. Now you are looking at the CD4+ subset within the lymphocyte gate. To show the graph on the next page (without gates), change the axes to CD45RA vs. L-Selectin (CD62L). You will see four populations (at least). Create a rectangular gate around these populations as shown in the figure on the next page. (Remember, to create a rectangular gate, hold down the option key as you draw the gate.) The CD45RA+L-Selectin+ cells are "Naive"; the rest are memory cells. You can name them M1, M2, and M3.

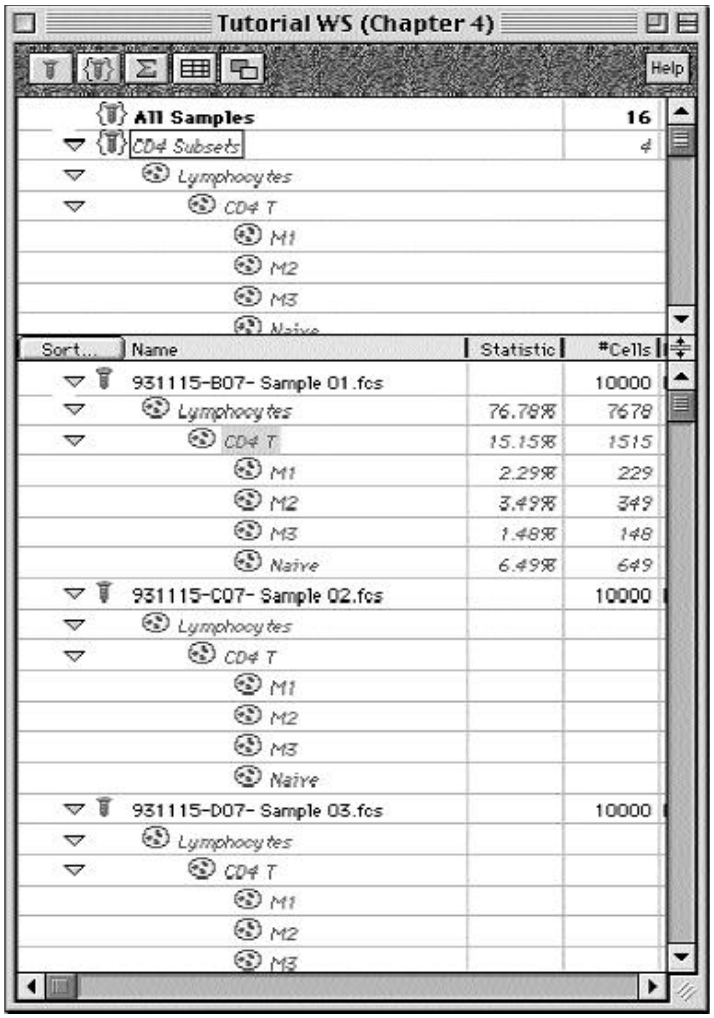


Your graph should now show these four gates. Close the ***Graph*** windows. If you hold down the ***option*** key while clicking on the Close-window box (the top left box in the ***Graph*** window), then FlowJo will close all open ***Graph*** windows. Your Workspace will show the subsets you just created, including the differentiation subsets of CD4 cells, which are a subset of lymphocytes, which are a subset of the entire sample (see below).

Note that the CD4 gate (and the CD4 subset gates) are *not* in green. This is because these gates have not been applied to the group, and therefore are not identical to a group analysis. They are drawn in plain black to denote the fact that these analyses are unique versions belonging to the sample.

To apply them to the group, click on the CD4 T subpopulation, and use the ***option***-drag action to move the gates onto the "Lymphocyte" subset of the group. (Note that if you were to drop it onto a higher level, a group name, the tree would be applied to each sample at the sample level – not to the lymphocyte gate. That is, the gates attach themselves to the row that they are

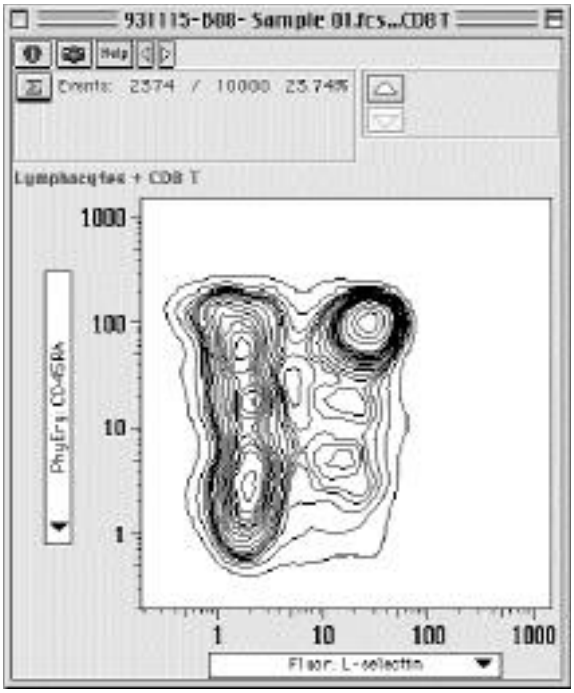
dropped on.) Your Workspace now shows these gates applied to all of the samples in the group, as shown here. As before, you can tell the program to recalculate the new populations and frequencies, or leave them as is, and FlowJo will calculate them automatically the next time you view, print or export these populations.



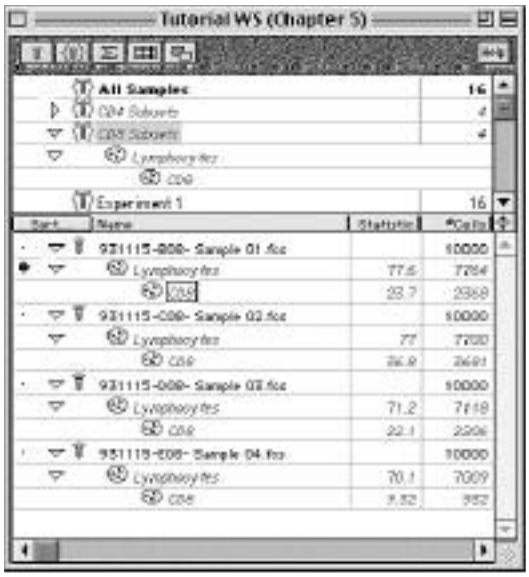
Lesson 5: Modifying Group Analyses

In this lesson, you will learn how to take advantage of previous work you have done (drawing gates, generating statistics) to save time when you do similar analyses. You will copy the gates you created for the CD4 cells to the CD8 cells. However, you will have to adjust them, then adjust the entire set of samples simultaneously, taking advantage of group analyses. Finally, you will finish all the group analyses for the entire sample experiment. You will then see how easy it is to modify the lymphocyte gate used by every sample with a single drag-and-drop action. Use your existing Workspace, or to better stay in sync with this text, open the workspace named "Tutorial WS (Chapter 5)".

Let's move on to the CD8 analyses, taking advantage of what we have already accomplished. Again, drag a lymphocyte gate (the single population only—don't press the ***option*** key) from any of the samples and drop it onto the CD8 subset analysis group. Select the group, and open the lymphocyte subpopulation from the first sample. Create a CD8 gate on the CD8 histogram (much as you did for CD4 cells). Then show the contour plot of CD45RA vs. CD62L. This plot is shown at the left.



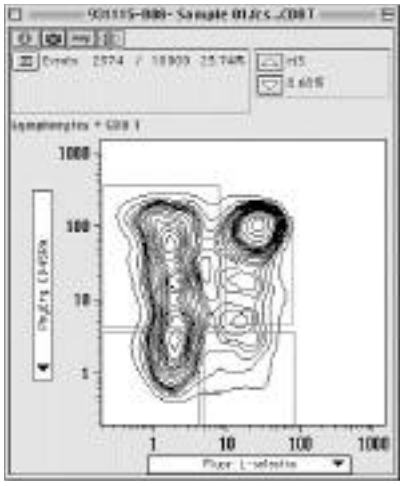
Move this window to the side where you can still see it, and click on the Workspace window to bring it to the front. Drag the CD8 gate you created onto the group's lymphocyte gate; the ***Workspace*** window will show the group's CD8 gate under each of the member



samples, as shown here, in the figure to the left.

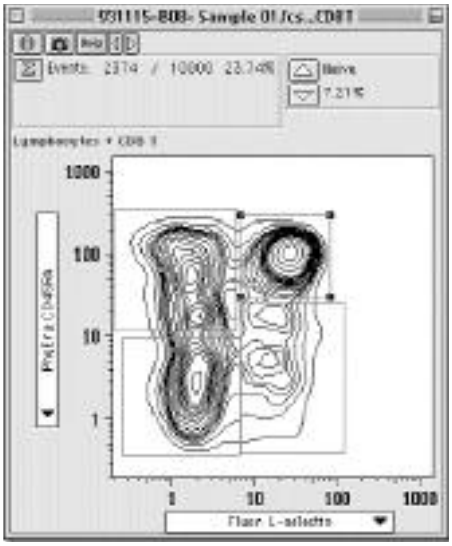
You will notice that the subsets of CD8 cells are similar to the subsets of CD4 cells. So, go ahead and copy the same gates you used for the CD4 cells onto the CD8 populations; you will then fine-tune them.

First, select the CD4 group again. Now, ***shift***-click on the Naive, M1, M2, and M3 subpopulations from one of the samples (see right). Drag this set of gates, and drop it onto the CD8 T gate, in the CD8 subset analysis group.



Watch the open ***Graph*** window as you do this: the four gates will appear in this window as they are attached to the sample! Again, FlowJo makes sure that every window that is open will reflect any changes you make. The Graph window appears as shown left.

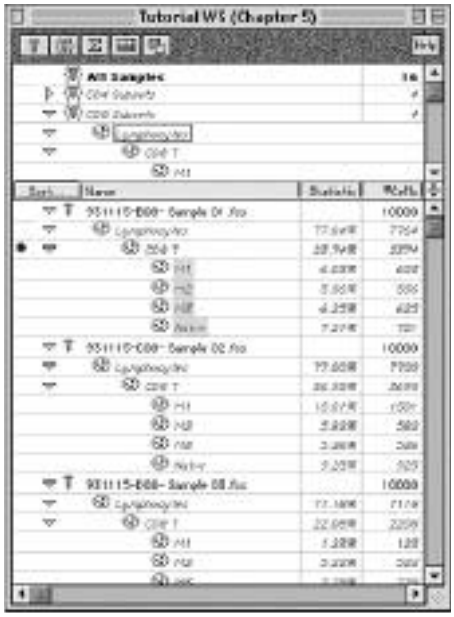
It is apparent that the gates are not positioned properly for CD8 T cells (these are the gates that were appropriate for CD4



subsets). In particular, they look as though they should all be moved up, since CD8 cells tend to express higher levels of CD45RA than CD4 cells. Click on each gate, and move them so that they more accurately enclose the populations (see left).

Leave this window open for now. Go back to the ***Workspace***, and open the graph for the CD8 subpopulation for Sample 02 (C08). It still has the gates copied from the CD4 stained sample.

Move this window off to the side as well, and watch it during the next operation. In the ***Workspace*** window, you will note that the subpopulations for the first sample are drawn in black – this is because you modified them and they are no longer equivalent to the versions in the group. However, these are the ones you want to apply to the group. Once again, this is easy to do: ***shift***-click the four subpopulations (Naive, M1, M2, and M3, under the first sample), and drop them on the CD8 gate in the group. In the ***Graph*** window for the second sample you will see the gates move to the new positions; in the Workspace window, all gates are now drawn in green, since all are now identical (see right).



You are now finished with the T cell analyses; go back to the PBMC subset group. ***Shift***-click the Lymphocyte

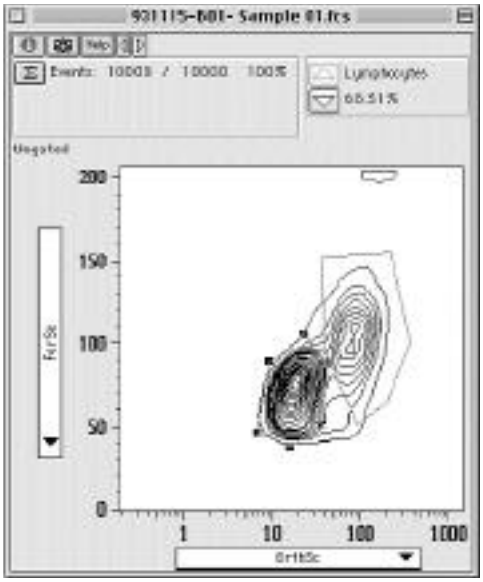
and Monocyte gates from the first sample, and use the ***option***-drag action to move the tree to the group node. This applies the analyses to the entire group (see right).

Now, if you click on the Experiment 1 group, you can look at all of the samples from this experiment in their full glory (below). Note that the assignment of color and style to the groups helps you identify which group any particular gate comes from.

As a final tune-up for the gating analysis, you will modify the lymphocyte gate and update all of the samples for this experiment to have the new version of the gate. It is important to see how the program's dynamic recalculation can be used to modify either single gates, or gates shared among multiple samples.

Open the very first sample, at the top level (the sample level – double-click on the top line). You will see the two gates, the lymphocyte gate and monocyte gate. Select the lymphocyte gate, and drag the individual handles so that it is "tighter" around the population (see the graph, top of the next page). Move all of the handles in to make this gate smaller.

The ***Workspace*** window will now reflect this modification by drawing the newly-



modified lymphocyte gate in black. (See the second line in the figure below.)

How can we update all of the samples for this experiment? All of the samples for this experiment belong to the group Experiment 1, the group that was created when you read in the folder of FCS files. So, you can use this group for analyses that apply to all samples. (Alternatively, you could create a new group, select all samples, and drag them to the new group to add them, and then use that group.) Drag the modified lymphocyte gate to the Experiment 1 group. Now look at the Workspace (shown on the next page). It may not look as you expect!

First, note that the lymphocyte gate that you dragged is now light blue, plain type – this is exactly like the Experiment 1 group. However, none of the other Lymphocytes gates are drawn in this color or style. Therefore, they are not the same as this gate! (Open another sample to convince yourself that it hasn't changed.) Why? Because of the second rule of group analyses: when you modify a group's analysis, or add a new analysis to a

group, then all samples receive that analysis *only if they don't already have an analysis of the same name.* All of the samples already had their own "Lymphocytes" gate. Therefore, FlowJo did not replace or modify them. This is a very important feature of FlowJo; this feature allows you to perform group-level analyses, but maintain individual sample variation. You can modify a single sample's lymphocyte gate, and still have that subset behave like every other lymphocyte subset.

Let's say you want all of the samples to have the same gate. FlowJo gives you an easy way to force all analyses to be identical. You do this using the **Unify Analyses...** item under the **Workspace** menu. If you invoke this command after clicking on a sample's subset, then only that subset is updated to have the same gate as the current group's version of the gate. If you invoke this command after clicking on the group's version of the gate (in the upper half), then all samples in that group are unified. So, click on the Lymphocytes subset under the Experiment 1 group, and select Unify Analysis... . FlowJo asks you to confirm this action (see below). If you click

on the Yes button, then your Workspace will soon look like the one here at the right.

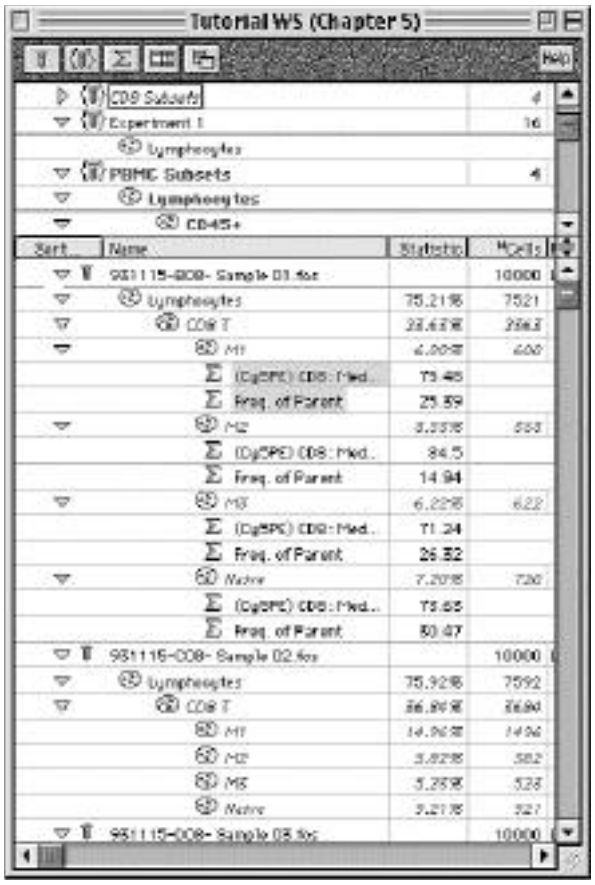
Note now that all Lymphocytes subsets are drawn in plain blue text, denoting that they are identical to the group's version. Open any sample's graph to verify that it has the right lymphocyte gate. If you then click on the group Experiment 1 and choose **Recalculate Samples** all gates and statistics for all samples in this experiment will be recalculated. After you have done this, select the CD8 T subset group.

One final lesson before moving on to tabular and graphical presentations: merging analysis trees. Let's assume that you modify an existing analysis tree on a sample, for instance, to add several statistics to different subsets. You would now like to propagate these changes to the whole group (or other samples) without having to drag each new statistic individually. FlowJo gives you the ability to easily merge analysis trees that are very similar, adding only the new or different analyses to the destination.

When you drag an entire analysis tree onto a population that already has parts of that tree, then FlowJo offers four options:

(1) **Cancel** (Do not change anything.)
(2) **Rename:** duplicates the tree (Renames the analysis tree you are copying and attaches it to the subset as a copy.)

(3) **Replace** the existing tree (FlowJo deletes the old analysis tree and replaces it entirely with the new one.)
(4) **Retain** the existing tree and add the new analyses (FlowJo only adds those analyses in the new tree that are not in the old tree.)



As an example, add two statistics to the first M1 subset. Click on the M1 subset, and click on the Add Statistic button. Ask for the Median of Cy5PE (CD8) and the Frequency of Parent statistic. Then, ***shift***-click these two rows in the Workspace, and drag them to each of the M2, M3, and Naive populations. Your Workspace will now look as shown to the left.

Here is an example where you have modified the existing tree (in sample B08), and wish to add the changes (the statistics) to C08. You could simply drag the two statistics nodes all the way to the M1 subset with C08 (resulting in the workspace shown on the next page). Then, you could continue this process. However, this is tedious, especially if you have more than a few changes. Instead, merge the trees. Click on the Lymphocyte subset under B08, and, while holding the option key (to drag the entire tree), drop it onto the C08 sample. Make sure you drop it onto the sample level – that is where it needs to be

attached. FlowJo now brings up the dialog shown below.

Here you can choose between the four options listed above. If you choose **Rename**, then FlowJo will rename the Lymphocyte analysis that you are dragging, and call it "Lymphocyte-1". (FlowJo has to rename it because two subsets with the same name cannot coexist).

Alternatively, you could select **Retain.** This instructs FlowJo to add any analyses that are different between the tree you are dragging and the existing tree, but not to modify the existing tree in any way. Thus, had you modified any of the gates in C08 (like the lymphocyte gate) so that they were different from the ones in B08, FlowJo would preserve those changes. However, the statistics would still be added in the appropriate places.

Or, finally, you can select **Replace.** This option instructs FlowJo to delete the existing Lymphocyte subset tree, and replace it with the tree you are dragging. Any sample-specific modifications that you had made for the analysis tree in C08 would be

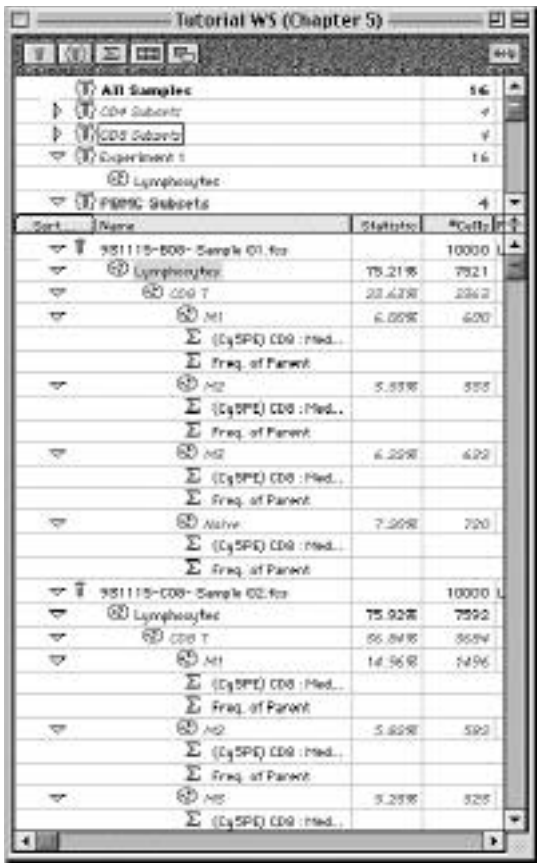
Duplicate Gate Resolver
How should gates with the same names be handled?
There are three different actions that might make sense here. You probably want to use Replace, but that could overwrite previous work.
Replace: The new analysis gates should replace existing ones of the same names
Retain: Do not modify existing analysis gates if they have the same name
Rename: Modify the name of the new analysis trees by adding a version number
Help
Cancel
Rename
Retain
Replace

lost, and the resulting analysis tree would be identical to the one in B08.

In this example, since all of the analyses in C08 are group analyses, they are identical to the analyses in B08 – so, in this instance **Replace** and **Retain** are essentially the same. Choose **Retain.** The Workspace will now reflect the newly-merged trees (below).

This is how you can easily add any modifications to an analysis tree from one sample to another – simply drag the whole tree! You can then choose to keep (the **Retain** option) or throw away (the **Replace** option) any differences between the common parts of the two trees.

You may have noticed that the ***Retain or Replace dialog*** window did not appear when you dragged analyses to a group, even when they already existed (for example, when you were modifying the Naive, M1, M2, and M3 subsets for the CD8 analyses at the beginning of this lesson). FlowJo assumes that when you drag an analysis to a group, you want to replace all of the existing analyses that have the same name. Then, every sample that has the group's version of those analyses will also get the new version that you have copied to the group. Dragging an analysis tree onto a group is essentially the equivalent of dragging the tree to every sample in the group, and choosing **Replace.**



Lesson 6: Tables and Layouts – Collating Data Output

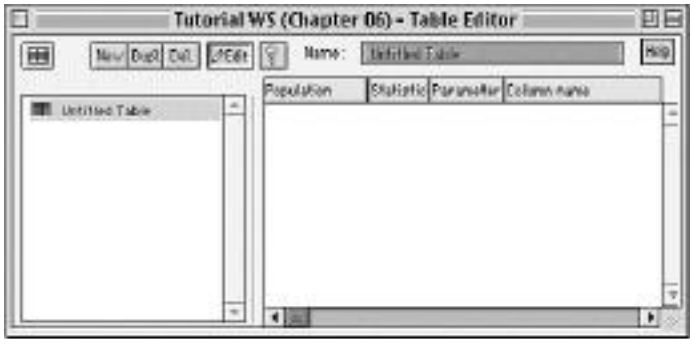
In this lesson, you will learn how to transfer any statistical analyses you have requested in the Workspace into other programs. You will be able to generate a table of statistics, bringing together any set of values from any combination of samples (using Groups to define the sample list).

FlowJo is not a comprehensive data presentation package. It provides the tools to analyze and graphically display flow cytometric data. Analysis tools such as linear regression, very complex graphical displays, etc., are more suited to programs specifically designed for those purposes.

However, FlowJo does provide tools to collate the output from multiple analyses so that you can import them into spreadsheets (tabular data) or into drawing programs (graphical displays). These tools include the Table Editor and the Layout Editor.

Use your existing Workspace from Lesson 5, or open the tutorial workspace named "Tutorial WS (Chapter 6)."

To open the Table Editor, you can click on the fourth button in the button bar (it looks like a table), or select the Table Editor from the Windows menu. FlowJo shows you the Table Editor window, and automatically the first table; change its name to "PBMC Subset Freqs." as shown here. (Click in the title field and type the new name for this table).



In this window, the left side holds the list of existing table templates. The right side will show the items in the currently-selected template.

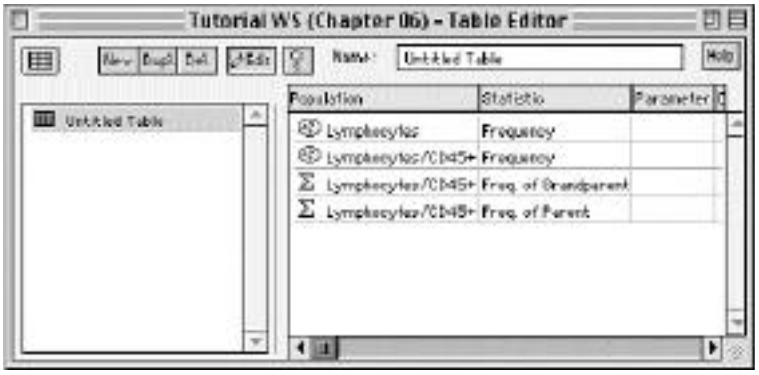
What is a **template**? When you define a table, you will add rows to a table template. Each row defines one statistic that you want to export, such as frequency, mean fluorescence of FITC, etc. When you create the table, FlowJo cycles through each sample in the current group and requests the particular statistic defined in the table.

Then, when you create the table and import it into another program, you will have one row in the table for every sample in the current group, and one column for every statistic in the table template.

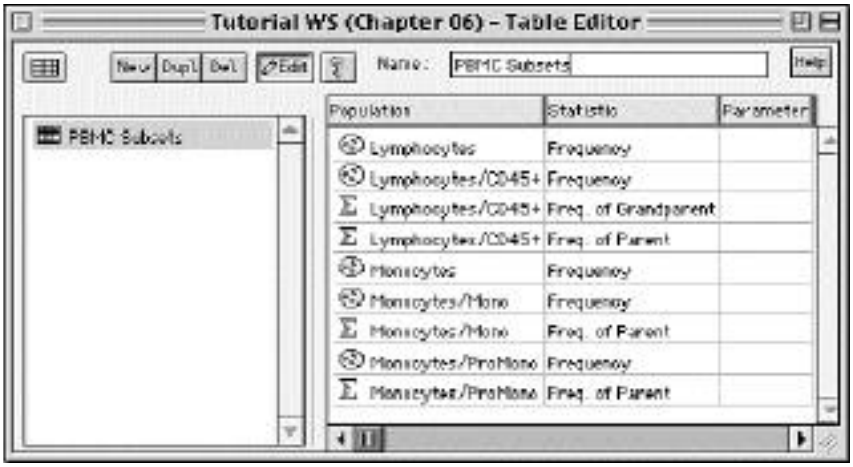
You may define as many table templates as you wish; you could use one for each different set of statistics. This one, as you may have guessed, will be used for generating a table of the major PBMC subsets analyzed in the first stain combination. Note that you can apply a template to any group; it doesn't matter which group was active at the time that you defined the table template. However, most likely your table will only be applicable to one group of samples.

Go back to your Workspace, and select the PBMC subsets group. From the first sample only, select the "Lymphocytes" and "CD45+" subpopulations, and the two frequency statistics under the "CD45+" subset. (***shift***-click to highlight the four rows.) Drag the highlighted rows and drop them into the right portion of the ***Table Editor*** window. The ***Table Editor*** will now look like it does here:

Just as you can use the ***option*** - drag action on analysis trees between samples, you can also use ***option*** -drag for transferring an entire tree to the ***Table Editor.***



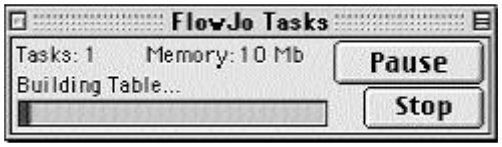
Click on the Monocytes gate in the first sample, and, while holding down the ***option*** key, drag it to the ***Table Editor*** window. You will note that all five of the analyses are added in order to the table (the ***Table Editor*** should now appear as shown below).

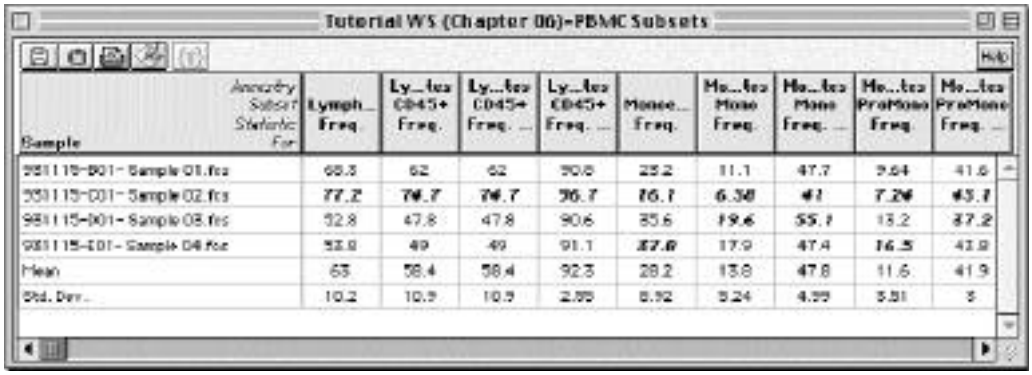


When you drag a subset, a gate, to the table, FlowJo assumes that the statistic you want is the Frequency (within the sample) of that subset.

To create the output table, click on the "table" button near the top-left of the window. FlowJo will display a new window in which the table will be shown. (If you analyze a large workspace, you may first see a progress monitor window as FlowJo builds the table; see bottom right. You can cancel the table build by clicking on "Stop"). As FlowJo calculates the values for the table, they are filled in. During the calculation process, FlowJo displays a "Progress Bar" near the top of the Table Window to show you how far along it has gone at any time. The finished table looks like that shown on the next page.

At this point, FlowJo has cycled through every sample in the current group (there are four), and

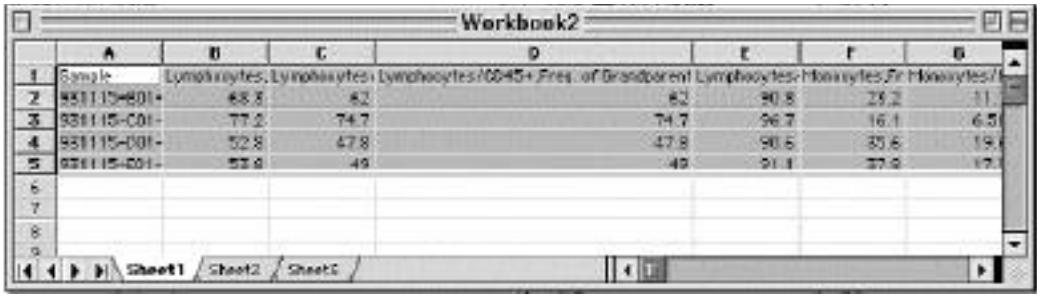




has calculated each of the nine different statistics you requested (on the applicable subsets). If a sample did not have the subset required, then there would be a blank entry in the table. (And, if the subset did not have the requested statistic node present, there would also be a blank entry).

The column heads show you the name of the subset's parent gates ("ancestry"), the name of the subset, the statistic, and the parameter on which the statistic is calculated ("For"). Each row in the table corresponds to a sample in the current workspace. You may resize the columns by clicking on a column divider (in the table header) and dragging left or right.

Optionally, you can show the Mean and Standard Deviation of the statistics at the bottom of each column. If they do not appear, check the Preferences window, under Tables & Layouts, and check the box marked **Show Summary Stats.** When the summary statistics are computed, the numbers in the cells are drawn in bold italic if they are greater than one standard deviation away from the mean, and in red if they are over twice the standard deviation away from the mean. This will quickly highlight outlying samples making it easy to use the table editor to identify samples that are significantly different from the others in the group.

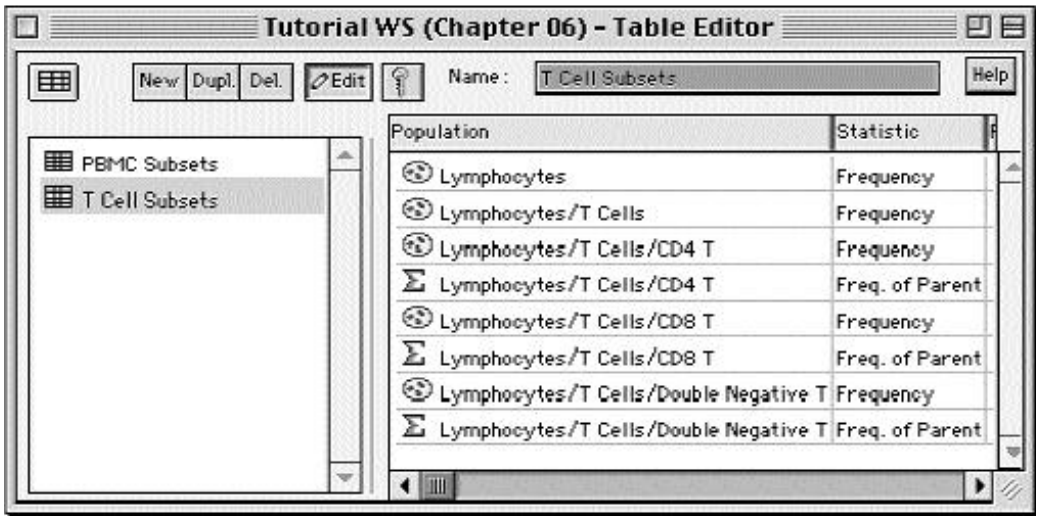


To export the table, click on one of the four buttons near the top left. The first button will save the table to disk (as tab & carriage-return delimited text data). The second button copies the table to the clipboard (and you can then paste it into a spreadsheet application; for example, the graphic on the this page shows the result of copying the above table into Microsoft Excel™). The third button prints the table; and the fourth button saves the table on disk and immediately launches an application of your choosing to analyze it.

The length of the column headings often results in long names (as exemplified in the Excel spreadsheet), often much wider than the space allowed for by the columns. However, you can keep the column names short by giving your own names in the final column of the Table Editor. In addition, there is also a preference to Use Short Stat Names in the Tables/Layouts section, but usually you'll be able to come up with more descriptive names than the defaults provided by the program.

The table window is always current. When you create a table, FlowJo goes through all the samples and makes sure they are recalculated according to the latest modifications. If you now go back and change the lymphocyte gate, apply that change to all of the samples, then that change will be immediately reflected in the table window. From the Table Editor, you can create new table templates, duplicate existing tables, or delete existing table templates using the buttons across the top. Click on New and name the new table "T Cell Subsets". From the

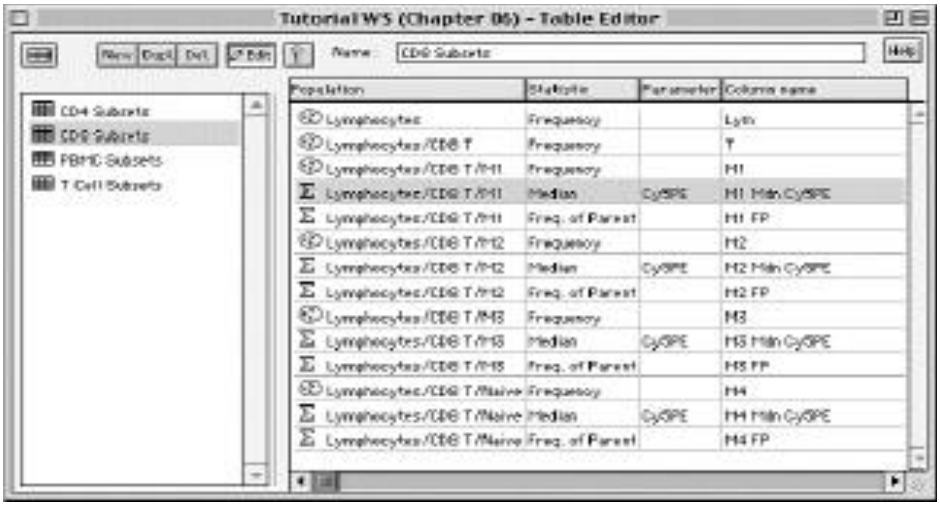
Workspace, select the “T Cell Analyses” group. Use the ***option*** -drag action and move the Lymphocytes subset from the first sample into



the Table Definition window; you will see all 8 rows: Create two more tables for the CD4 Subsets and CD8 subsets. Again, ***option***-drag entire analysis trees to bring everything into the table. Note that you can change the order of the table entries by clicking on any entry and dragging it to another place. You can also delete a table entry by selecting it and pressing the ***delete*** key. After creating the last table, the Table Editor window should look similar to the one shown on the top of the next page.

Notice in that picture how the column names were assigned for each of the columns generated in the CD8 Subsets table. These names will appear both within FlowJo’s tables, and when the data is exported to other applications.

You can now apply these table templates to the appropriate groups and get output tables with specific sets of statistics: just select any group and click on the “Table” button.



Remember, you can apply a table template to any group; this could be useful for separately analyzing different experiments (that you have grouped separately). If you apply a table to a group that has no gates as requested by the table, you will get a lot of empty values!

Tables can also be added to Layouts (graphical layouts) using the ***Layout Editor***. Simply drag a named table (from the left portion of the ***Table Editor*** window) and drop it in the Layout Editor and FlowJo will create a live table that updates as you create batch graphical layouts!

Table definitions are saved with the Workspace. When you re-open the Workspace, you can recalculate the tables. Tables can be applied to any group; you can, in the future, read more samples into the Workspace and apply the table to those samples. Table templates are a useful "batch analysis" tool; they allow you to quickly collate many statistics from many different samples for analysis by other programs.

FlowJo creates tables in the background, letting you perform other analyses while it computes statistics. You can even have it start many different tables computing simultaneously, then go back and analyze more data. FlowJo schedules the calculations so efficiently that it's actually faster if you create multiple tables & layouts simultaneously!

Lesson 7: Creating Simple Graphical Layouts

In this chapter, you will learn the fundamentals of the Layout Editor. You will be able to generate layouts with multiple different graphics, text items, lines, etc., and you will learn how to create overlays of graphs. In Chapter 8, you will learn how to create Batch Reports, where the layout is applied to all of the samples in the workspace. Finally, in Chapter 9, you will learn a few of the features of the Layout Editor that let you create complex multi-sample layouts, including statistics and graphs from multiple tubes in a panel.

This lesson continues with the Workspace document you have finished from Chapter 6; alternatively, you can open the workspace named "Tutorial WS (Chapter 7)".

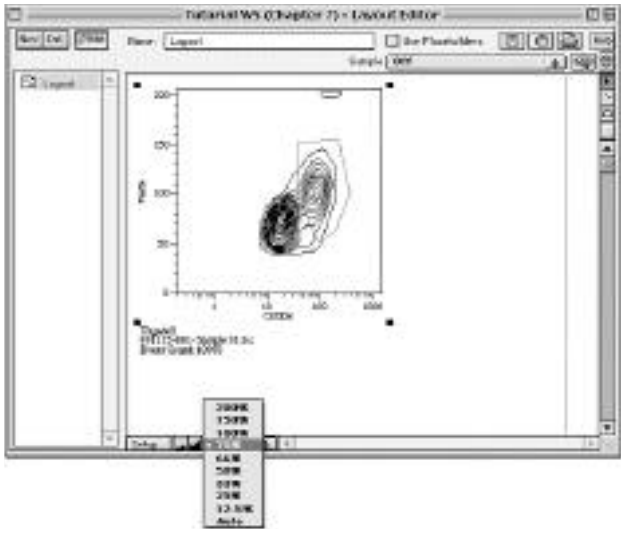
To open the Layout Editor, either select **Layout Editor** from the **Windows** menu, press ⌘-L, or click on the "Layout" icon in the row of icons at the top left of the Workspace window. You will be shown an empty layout editor.

In the ***Layout Editor*** window, the left portion of the window is a list of all of the layouts you have created for this workspace. You can have as many as you wish; you will probably want to create different layouts for different purposes. (For example, in the Demonstration Workspace shown in the Introductory chapter, there were Layouts for generating Patient Reports, for verifying scatter gates, etc.) You can name the layout by clicking in the "Name" field near the top of the window and typing a new name.

To add a graphic to the Layout, click on any subset in the workspace and drag it into the layout view. For now, click on the top-level (ungated sample) "931115-B01..." and drag it into the Layout View. You will immediately see a graphic corresponding to the subset. (The default graph that is shown is the same as how the subset was last viewed). In addition, FlowJo creates an "Annotation" text box below the graphic that

contains some pertinent information. Most of these options have a default value in the "Layout Preferences," so that you can tailor the information added to your graphs in the ***Layout Editor.***

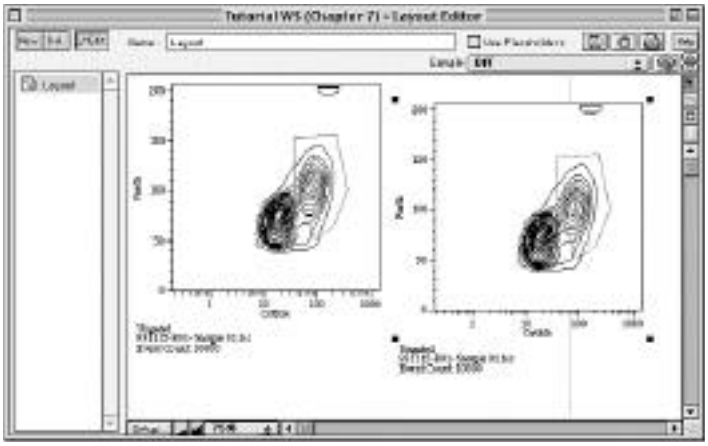
Any graphic item can be resized or moved just by clicking and dragging. To resize, click and drag on one of the four handles at each corner. (If you hold down the ***shift*** key while resizing , the graphic maintains its original aspect ratio). You can also change the magnification of the view by clicking on the Scale popup menu near the bottom of the window (see example, right).



One of the important aspects of the ***Layout Editor*** is that it is live. This means that any time you change or move a gate or modify an analysis in the ***Graph*** Window, FlowJo will automatically update the Layout Editor if needed. Thus, you can use the Layout Editor to provide instantaneous feedback for gating operations, where you can simultaneously view many different subsets (or even multiple views of the same subset) while moving a gate used to define that subset.

To create another view of the same subset, you have three choices: (1) drag the same subset from the Workspace window into the Layout Editor again; (2) select the first graph in the Layout Editor, and do a **Copy** and **Paste** operation; or (3) select the first graph and hold down the ***option*** key while you drag it – this creates a copy of the object and then lets you position it. For now, ***option***-drag the first graph and move it over to the side (see next page).

You will note that the second graphic is identical to the first. To change how it looks, double click on the graphic; FlowJo shows you the ***Layout Item Definition*** dialog as shown below. From this window, you can specify exactly

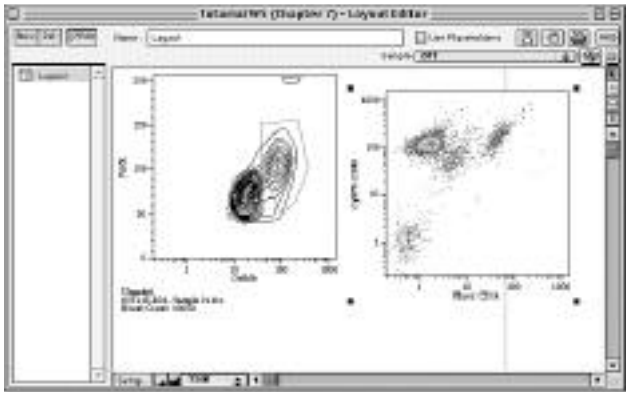


how you want the graphic to appear. As shown in the example, change the X and Y axes to CD14 and CD45, and change the graph type to "Pseudo-color".

Now click on the Annotate tab near the top of the window. This shows a

different set of options that control what appears in the graphic (see right). Unselect the checkbox **Show Annotation**: this will remove the annotation text box below the graphic. Click on **Set**, and the

Layout Editor should appear as follows. (On-screen, it may appear that there are fewer events; this is due to the reduced magnification in the window. Verify this by selecting 100% in the magnification window. When you copy or print, all dots will show up).

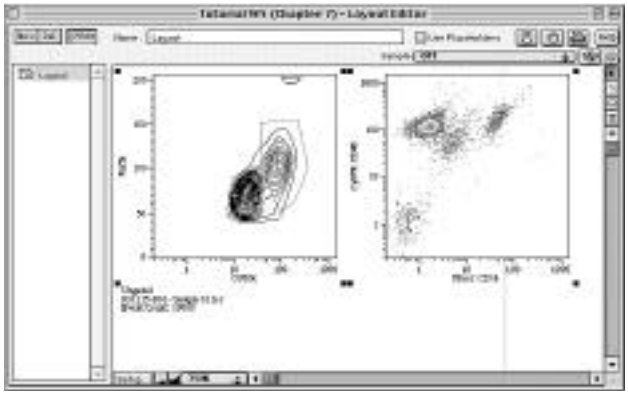


FlowJo provides many features of drawing programs, such as the ability to align multiple objects. To align the two graphs, select them both (***shift***-click on the graphics, or, use a marquee selection tool by clicking and dragging to encompass both graphs), and then choose **Alignment...** (⌘ - K) from the **Layout** menu. FlowJo brings up the ***Align Objects*** window shown left.

You may choose to ***Align*** or ***Distribute*** objects in either or both dimensions.

For now, choose to **Align by Top,** and click on **Set.** The ***Layout Editor*** will reflect your changes:

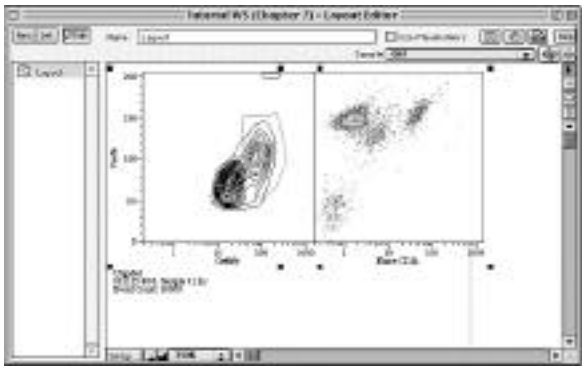
Sometimes, adjacent graphs have exactly the same Y axis (or X axis).



FlowJo lets you remove the axis labels and align the objects to be adjacent to each other – easily creating compressed graphical presentations suitable for publication. Double-click on the right-most graphic, and, under the **Annotate** options, choose to hide the Y axis (see right):

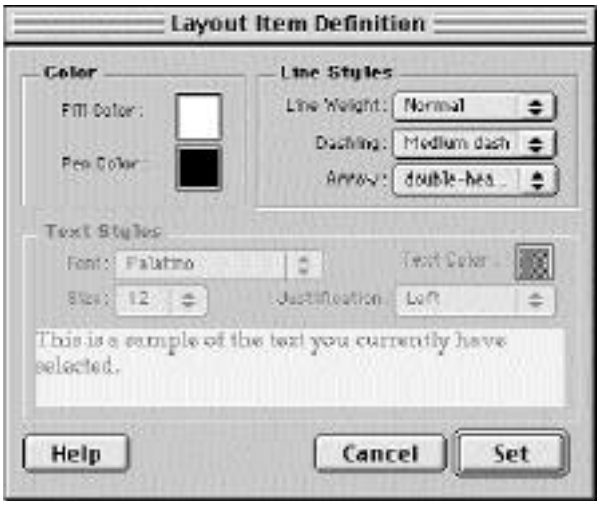
Now, select the two graphs and choose **Alignment...** again. Now you wish to align them by the top edges, and the align them adjacent to each other (Align by Width). In addition, you can ask FlowJo that it aligns graph items by the position of their axes (rather than by the position of enclosing rectangle: check the Align graph items... box). The ***Align Objects*** window should look as shown in the middle figure at right:

Now the ***Layout Editor*** shows the two graphic items side-by-side, sharing a common Y axis. (Of course, in this example, the Y axis is different for the two plots; however, FlowJo still lets you put them side-by-side – giving you complete flexibility in displaying graphs). The layout will now appear as shown on the bottom right:

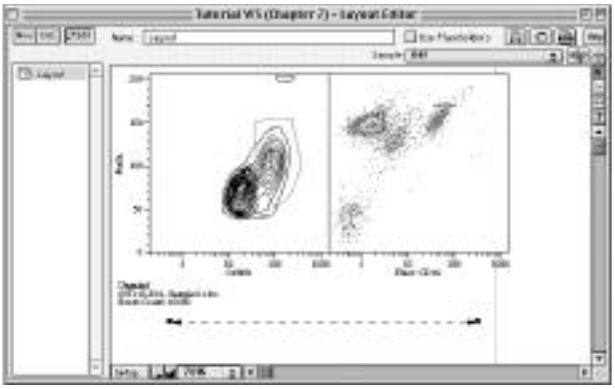


FlowJo's ***Layout Editor*** provides a few simple drawing

tools to elaborate your graphical reports. These tools can be selected by clicking on one of the tools near the upper right edge of the window (as shown here). You can choose a ***Line*** tool (to draw lines and arrows), a ***Rectangle*** tool (to draw filled or unfilled rectangles), a Text tool (to create text objects), or a ***Grid*** tool to create grids (tables). Click on the ***Line*** tool, and draw a line by clicking and dragging underneath the two graphics. (If you hold down the ***shift***-key, the line is forced to the nearest 45 degree angle). If you now double-click on the line (or, select the line and choose Get Info... from the **Layout** menu), you can change the style of the line. FlowJo shows you the ***Lay - out Item Definition*** window as shown to the left.



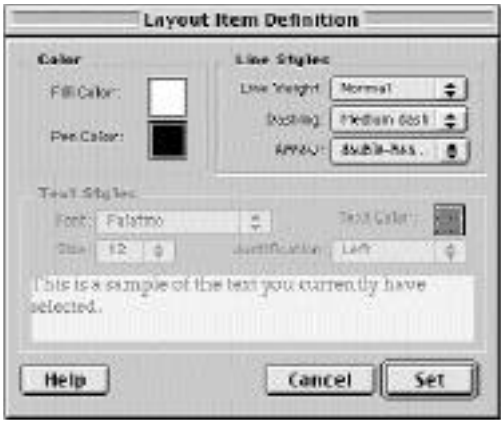
Change the line to be dashed by selecting **Medium dash** in the **Dashing** popup menu; and select the line to have arrows at both ends by choosing **double-headed** in the **Arrow** popup menu. If you wanted, you could also change the Pen Color for the line. Click on the button marked **Set.** The dialog box will go away. Your ***Layout Editor*** should now show the dashed arrow, and look something like the one shown here:



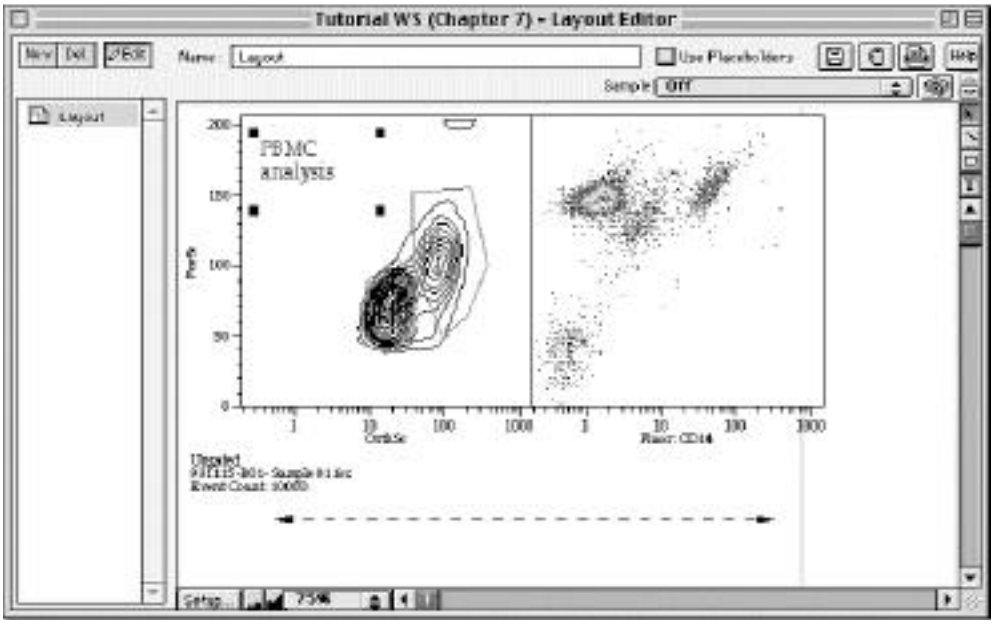
To create a text object, click on the Text tool, and then

click anywhere in the Layout View. (To create a text box with defined boundaries, click and drag the boundaries you want to have). FlowJo displays a new Text Editing window, into which you can type any text. (You can also drag statistics from the Workspace and add Keyword values by clicking on the Keyword menu. These items are "live" and will change as you create batch layouts for multiple samples!). Type "PBMC Analysis" into the window, and click on **OK**.

You can change the font & style of the text in a text box by selecting the text box and choosing **Get Info...** from the **Layout** menu. (Note that if you double-click on the text box, you will go into the Text Editing mode, where you can change the contents of the text). Select **Get Info...** to change the text color and font size as shown at left. Select the Text color to be red, and the size to be 18. (Note: the fill color is applied to the whole bounds of the text box; the pen color is applied to the box drawn around the text box – which is drawn only if the "Line Weight" is set to something other than "None".) Click on **Set** to confirm the changes.



Your ***Layout Editor*** should appear as the example on the next page.



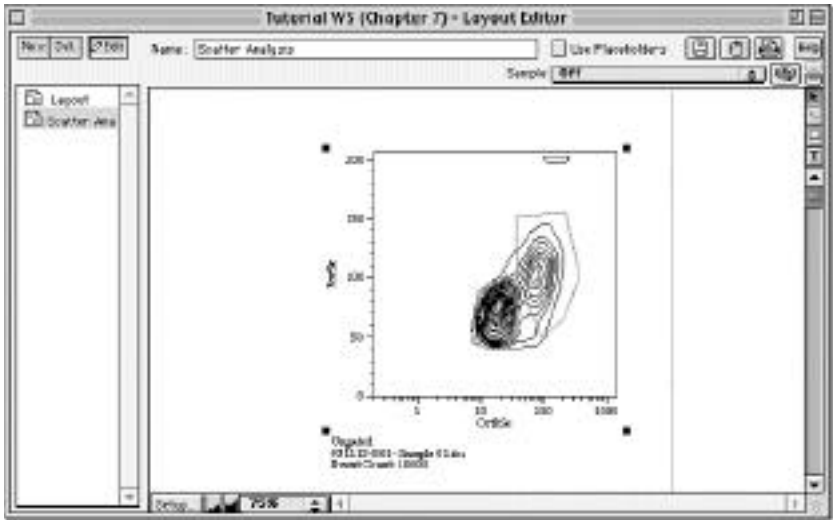
Layout Graphics can be easily exported, saved, or printed. To copy a subset of the items in the layout, select the ones you want and choose **Copy**; now you can **Paste** into any drawing program. You can export the entire contents of the ***Layout Editor*** window by selecting one of the ***Action buttons*** shown left (they are near the top right corner of the window).

The first button is an icon of an application. This button will save a PICT file to your disk, tell the Mac to launch another application (as set in the Layout section of the Preferences) and direct that application to open the file. The second button is a disk: if you click on this button, the graphics are saved as a PICT file on your Macintosh; any drawing program can open this file. The third button causes the graphics to be copied to the Clipboard; you can then paste them into any drawing program. Finally, you can choose the Printer icon to print the graphics directly to your printer. If you choose to Print, you may wish to change the **Page Setup...** (from the **File** menu or by clicking on the **Setup...**

button) to orient the page and change the magnification as you desire. (The light gray lines drawn in the Layout View represent page boundaries for printing; you can use these as a guide to set the ***Page Magnification*** appropriately).

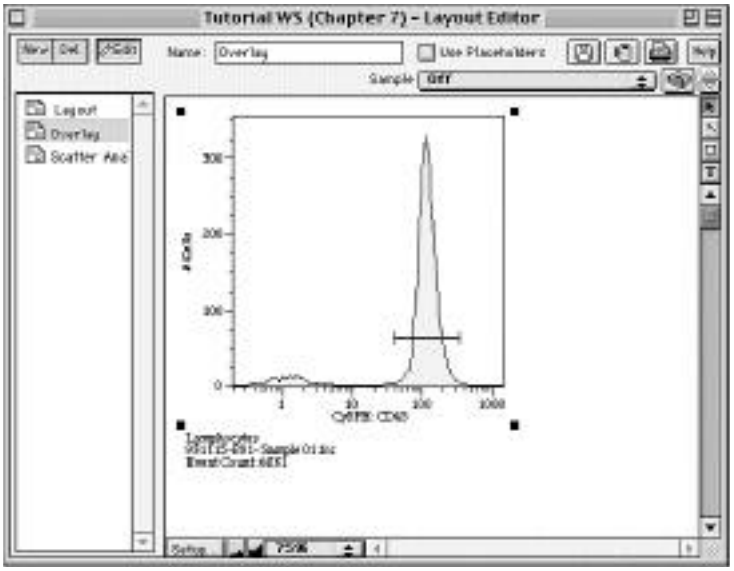
You can also copy items from layout to layout (within the same workspace). Click on the first graphic only, and choose **Copy** from the **Edit** menu (or press -C). You will now create a new layout and paste that object into the new layout.

As noted before, you can have as many different layouts in each Workspace as you wish. You will now create two more for this workspace. To create a new layout, click on the New button near the top left of the window. (To delete a layout, click on the Del button). Click on the New button and name the new Layout "Scatter Analysis". Now select **Paste** from the **Edit** menu (or press - V); the previous graphic will now appear in the new layout:

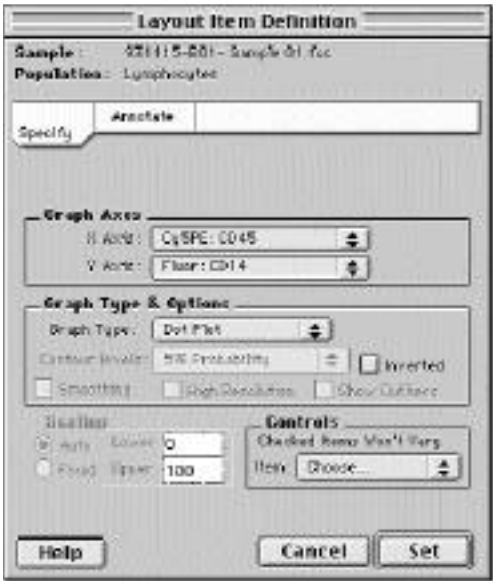


We will use this particular layout later. For now, let's create a third layout to demonstrate how to create overlaid graphs. Create a new

layout and name it “Overlay”. Drag the first “Lymphocytes” subset (from “931115-B01...”) and drop it in the view. You should have a view of the CD45 histogram as shown here:



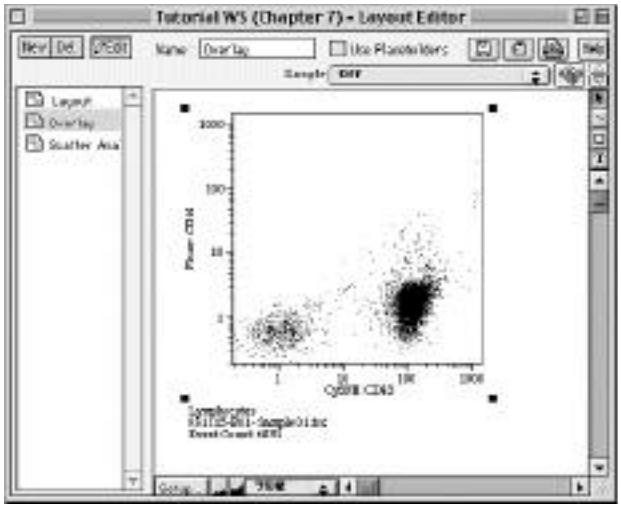
Double-click on the graphic, and change the graph specification to be Dot Plot of CD14 vs. CD45. The ***Layout Item Definition*** window should appear as shown below.



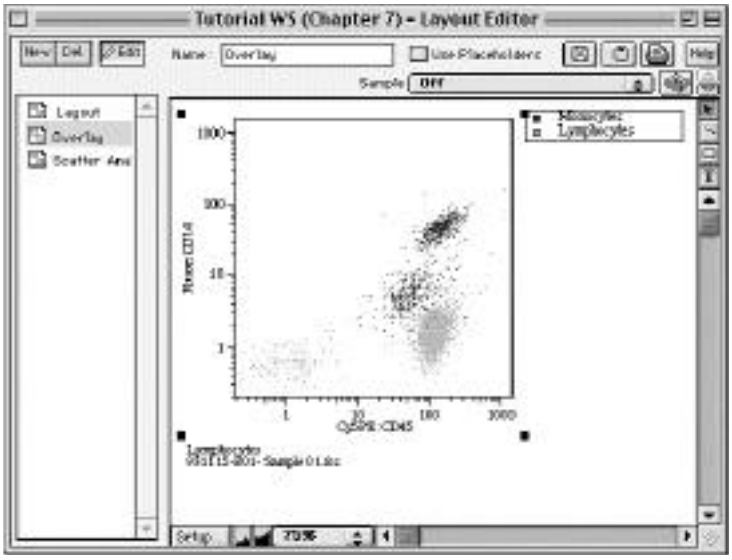
Click on **Set**, and the Layout View now shows you a Dot Plot, as shown on the facing page.

Any graphic item can be made into an overlay by dragging any other subset and dropping it onto the overlay. You can overlay different subsets from the same sample, or overlay plots from different samples. For now, select the “Monocytes” subset from the same sample and drop it on the graphic (the graphic will highlight to let you know that you can drop it to create an overlay). You should

now see the multi-color dot plot shown at the bottom of this page.

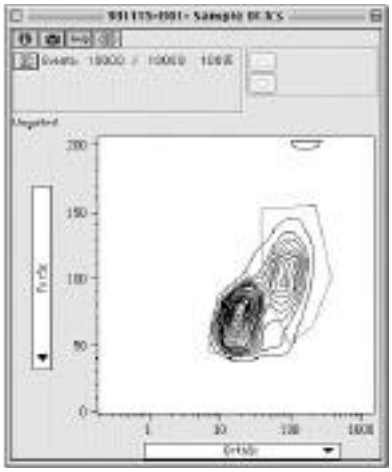


FlowJo is drawing the two dot plots, one for each subset, in the same graph. In addition, it automatically creates a ***Legend*** for the overlay, shown to the right of the graphic. You can change the color of any subset by clicking on the color box next to that subset in the ***Legend*** text box. You can change the order in which the dots are drawn by clicking on any item, and moving it up or down in the list. Finally, you can delete an item from the overlay by holding down the option key while clicking on one of the subsets (the cursor will temporarily appear to be a trash can when this happens). You can overlay an almost unlimited number of different subsets on the same graph.

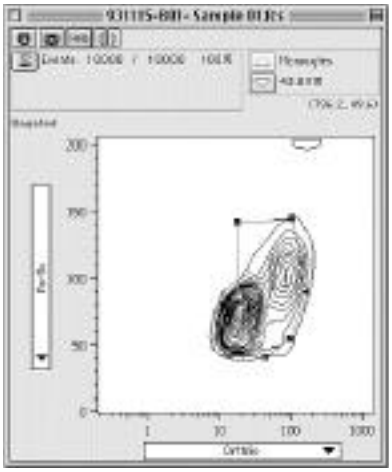


(**Note:** you can choose to show the Legend box for any graphic, even if it is not an overlay, from the Annotate preferences in the Layout Item Definition Window. Then you can set color and line styles for single graphs just like for overlay graphs).

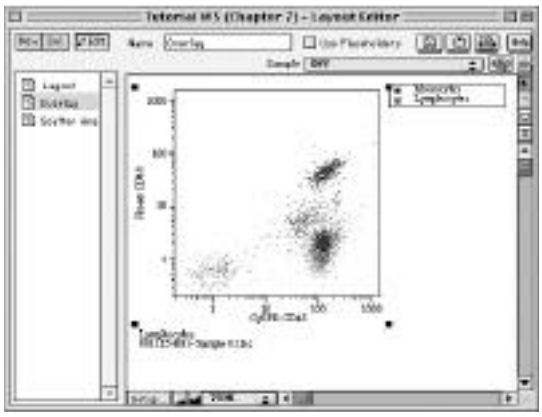
As noted before, all Layout Graphs are "live." This means that if you change a parent gate for one of the subsets, the graph is automatically updated. To see this in action, switch your attention back to the workspace, and double click on the "931115-B01..." sample node. You should see the graph to the right, defining the gates for the "Lymphocytes" and "Monocytes" subsets:

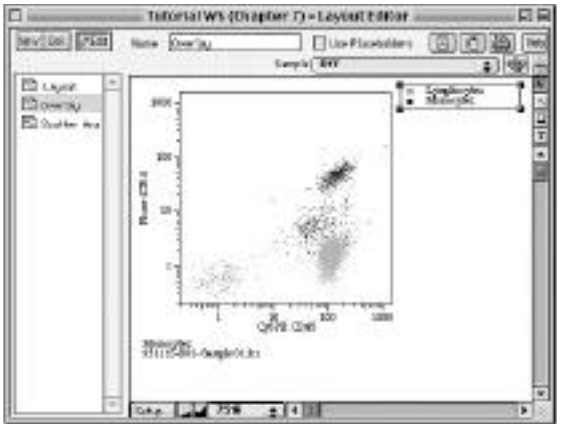


Click on the "Monocytes" gate (upper right gate), and move it around – perhaps, as shown in the second graphic, to overlap with the "Lymphocytes" subset.



Note that the Layout Editor responds by updating the blue dots (corresponding to Monocytes). The order in which the subsets are drawn can have a significant effect on what the graphs look like. To change the order, click on any subset in the Legend, and drag it up or down (the cursor changes shape to let you know what is happening). For example, click on the Lymphocytes subset, and move it to the top. Note that the area which has both blue and red dots now appears to be completely red, because the red population is on top (i.e., drawn last)--see the example on the next page.

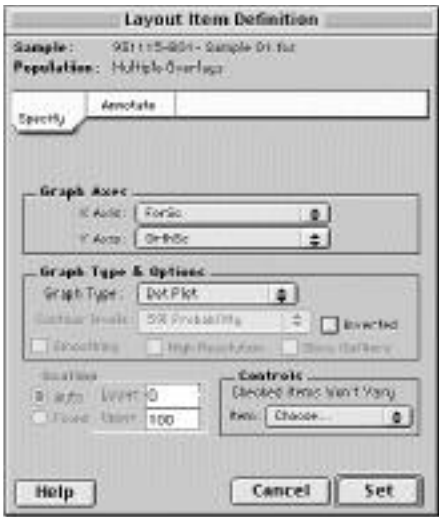


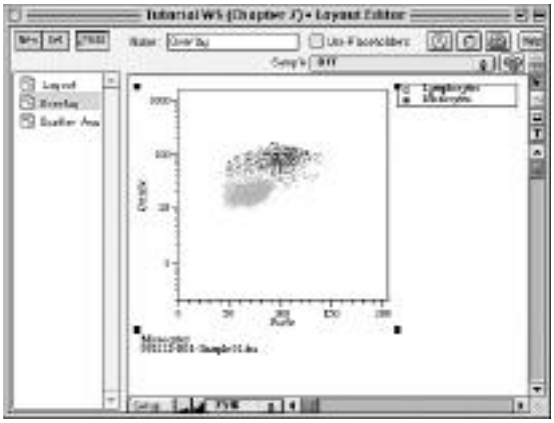


Once you create an overlay graphic, you can easily change its appearance (axes and plot style) – just like any other graphic. Double-click on the item; you will be shown the ***Layout Item Definition*** window. As shown in the example below, change your graph to show OrthSc vs. ForSc (side vs forward scatter).

Now, the ***Layout Editor*** Window should look something like the image below. See that the dot clusters reflect the gates which defined their colors.

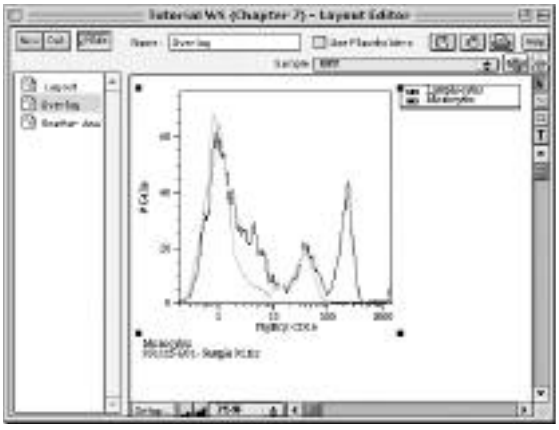
Double click on the graphic again, and choose to display a histogram of CD16. FlowJo now shows you a histogram overlay, like that shown at the top of the next page .



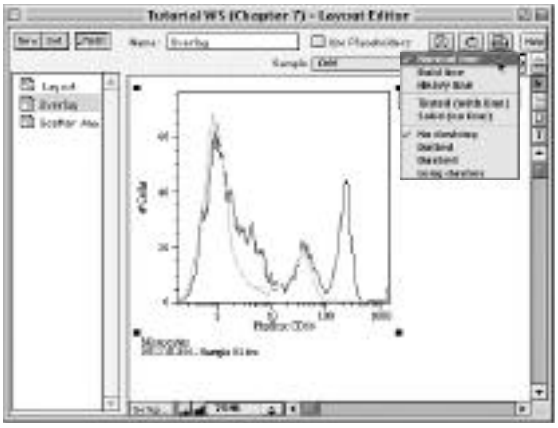


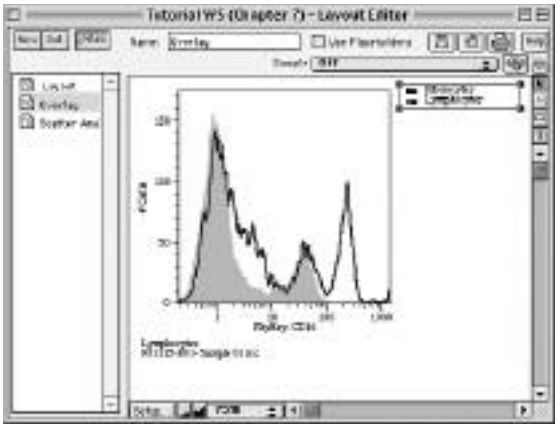
Note that the legend now has an additional set of controls right next to the color boxes. These are popup-controls that let you set the histogram line and fill style for each subset. If you click on the line control next to

the red box, you should see a popup menu as shown in the second graphic to the right:



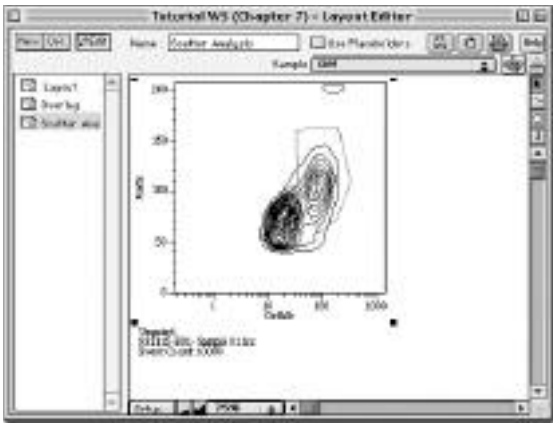
Here you can select the line weights (**Plain, Bold**, or **Heavy**), if the histogram should be filled (**Solid**), or if it should be drawn as both a line as well as filled. Select **Solid**. Then click on the control next to the Blue (monocytes) color box, and select **Bold**. This will edit the line weight and the fill color of the two lines in the graph. Your Layout should now show the overlay histogram as shown in the bottom graphic. Note that if you change the order in which the subsets are drawn, you can have a substantial effect on the output. To see this, click on "Lymphocytes", and drag it up so that it is first in the list.





To finish up, switch back to the "Scatter Analysis" Layout. You will note that the monocyte gate is still "incorrect." To correct the layout, open the "931115 B01..." sample again, and move the monocyte gate to the correct position. This will cause FlowJo to automatically recalculate the affected population and all child populations (which are affected indirectly because the gate determines all child populations. The picture to the right reflects the fact that the gate moved.

There are many options to changing how your graphs appear. You can choose to have FlowJo show gates drawn on a population (as it is here), or leave them off (**Show Descendent Gates**). You can create a ***History plot***, where FlowJo shows you not only the graph for a subset, but the plots of all of parent populations as well.



FlowJo gives you enormous flexibility in designing customized layouts, so that you can quickly and easily generate high-quality publication and presentation graphics.

Lesson 8: Creating Batch Graphical Reports

In this chapter, you will build on what you learned in Chapter 7 to generate graphical reports for entire experiments. You will learn how to cycle the Layout through different samples in the Workspace and how to create a combined output for printing or export of every graph for every sample.

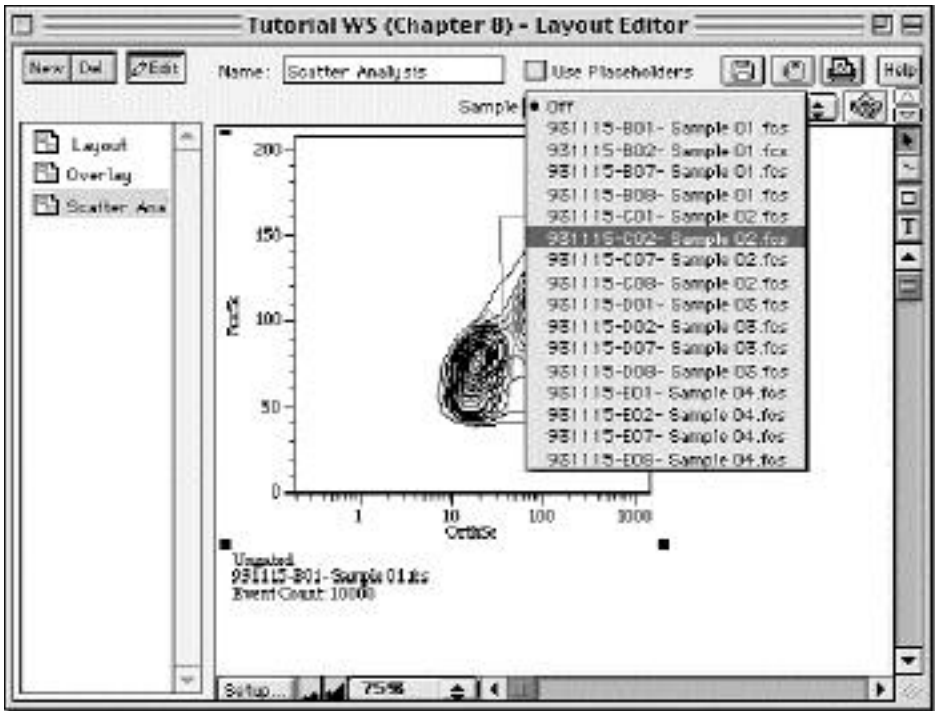
This lesson builds on the Workspace you have finished from Chapter 7; alternatively, you can open the workspace named "Tutorial WS (Chapter 8)." Open the Layout Editor, and select the "Scatter Analysis" Layout.

When you drag items into the Layout View, FlowJo by default shows you the desired graph for the sample from which you dragged the subset. However, FlowJo can show you the corresponding graph from any sample in the current group. To do this, you must select an "Iteration Value" corresponding to the desired sample.

First, what is an ***Iteration Value***? In order to perform batch processing, FlowJo cycles over every sample in the current group (i.e., whichever group is selected and displayed in the workspace). Thus, in the All Samples Group for this experiment, there are 16 samples, and there are 16 different ***iteration values*** for the group: one for each sample. In the "CD4 Analysis" group, with four samples, there are only four ***iteration values***, corresponding to the four samples in that group. Normally, there is a one-to-one correspondence between an iteration value and a sample in the group. (In Chapter 9, you will learn how to iterate by other criteria to create more complex reports.)

The Current ***iteration value*** is displayed and selected in the Iteration Popup menu, which is just the below the Help button in the ***Layout Editor.*** Whenever you create a new Layout, the current Iteration state for that view is set to **Off**. When Iteration is off, then all of the graphs shown in the Layout are identical to the ones you dragged and dropped into the View originally–FlowJo doesn't care what the current group is.

If you click on the iteration popup menu, you will see a display such as that below:

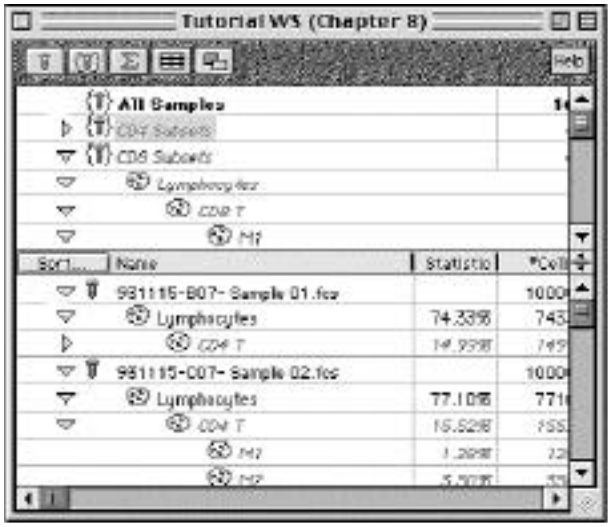


Because the current group is **All Samples**, FlowJo shows you the 16 different possible Iteration Values—one for each sample in the current group. You can see all of these values in the Popup menu. Select one of the values from this popup menu (for example, the "931115-C02...", as shown above).

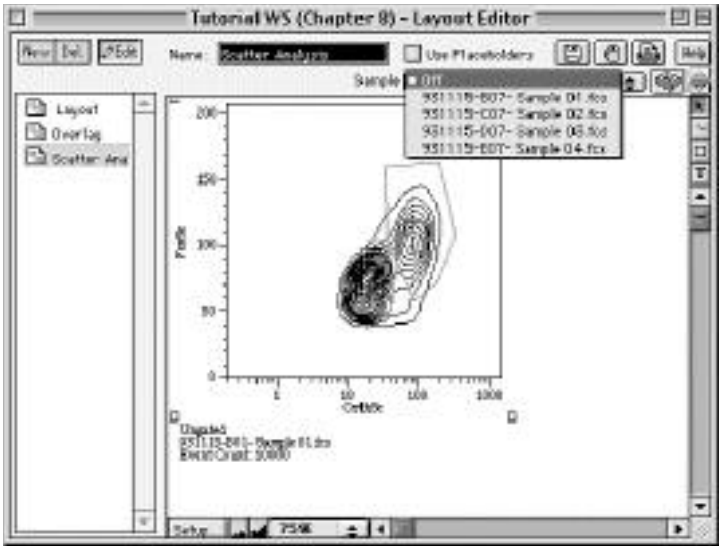
By selecting a specific ***Iteration Value***, you direct FlowJo to display the graphs in the View as they look for the sample you just selected. Therefore, the graphs you now see are those for the "931115-C02..." sample. (Note that if this sample did not have any of the subsets displayed in the Layout, then FlowJo would show an empty ***Placeholder*** for the graph.) Note the pair of arrow controls at the right edge of the window (right

below the Help button). You can use these to increment or decrement the current iteration value by one; i.e., you can use them to cycle through successive samples in the current group.

Switch back to the Workspace, and select the "CD4 Subsets" group (see right). In this group, there are only four samples.



Now, if you select the iteration popup menu from the ***Layout Editor*** window, you will only be given a selection of four different samples to choose from (see below). Again, this is because whenever iteration is not off, FlowJo looks through the current group to decide what possible samples can be displayed. Note that if you construct a Layout for a specific group which has unique sets of gates and statistics, then the layout may not operate as desired on other groups which don't have those gates! Once again, if you select **Off** for the current iteration value, then FlowJo shows only the original graphs for this layout View (the ones you



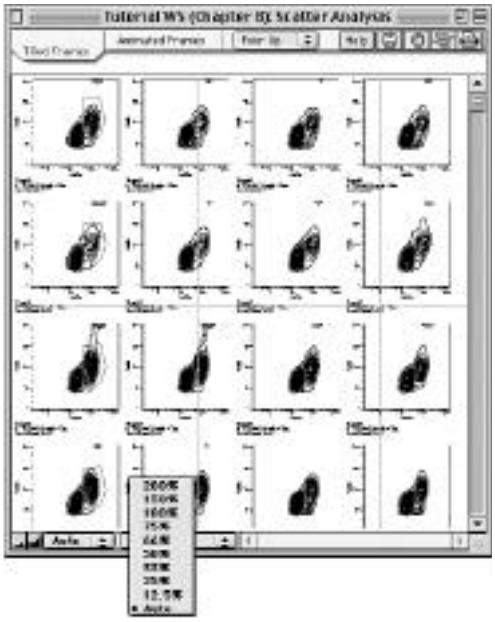
dragged and dropped into the view), irrespective of what the current group is.

Understanding how FlowJo generates a graph for any given Layout Item during batch processing is very important.

FlowJo draws a graph in the ***Layout Editor*** when all of the following criteria are met: (1) The Placeholders checkbox is not checked. (2) the current sample (i.e., the current value of the ***Iterator***) has the parameters that are displayed in the graph (like Forward Scatter, FL3, FL4, etc.) and (3) the current sample has a gating tree that has exactly the same subset as what is desired in the graph (i.e., if the graph was dragged from a CD4 subset of Lymphocytes, then FlowJo looks for a CD4 subset of Lymphocytes in the current sample). These two criteria cannot be changed. There is a fourth criterion that can be overridden by the Preferences, and that is (4) the current sample has the same stains (like CD3 or CD4) for each of the parameters as does the original sample. In the **Preferences**, under **Layout**, you can relax this constraint. This may be necessary if, for example, you have not properly annotated your samples.

So far, you have only looked at the Layout Report for individual samples, one by one. To look at graphs for all samples at once, click on the ***Batch Generation*** button (left), which looks like a stack of papers next to the Iteration Popup menu. This dialog will let you set the options governing how the report will look. You can set the format of the report, the group that will be processed, the geometry of the rows and columns in the report, and other options to set FlowJo to "do the right thing" without asking you every

time. Select the Tiled Report, set the geometry to 4 columns, and click on Create.



When you generate a Batch Output, FlowJo will start with the first ***iteration value***, and generate a ***frame*** for that particular sample. FlowJo then continues to generate a new "Frame" for each successive "Iteration Value" until it has exhausted the current group. These Frames are now shown to you in the ***Tiled Report*** window, such as that shown here.

Batch Layouts can be displayed in four different modes. The first type will simply create a new layout in the layout editor with all of the iterated samples shown. This is the most flexible way to generate a report, as you can now edit the layout further, adding titles or annotations of specific graphs, or by removing graphs that are not interesting.

The second mode is a Tiled Report, in which you get a new window containing each of the tiles laid out on a special page grid.

The third mode of reporting is a Web Report, where FlowJo creates a folder containing each frame as a separate graphic, and creates an ***index.html*** file to easily view the graphics from the World Wide Web.

The final form of reporting is a Quicktime Movie, where each set of graphs becomes one frame of an animation. You can play the movie in FlowJo or any movie viewer and cycle through all of the graphics.

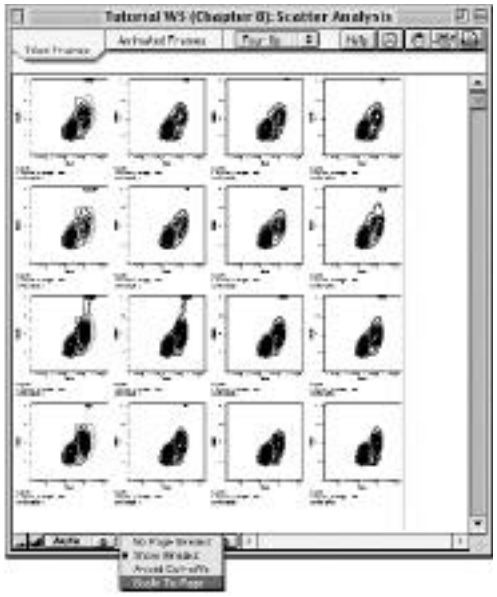
All of the different views generated in these batch reports support printing, saving to disk, copying to the clipboard, and creating Web pages.

Back to the **Tiled Report** view. There is a set of controls near the lower left of the window that control the magnification of the view. You can scale to any percentage, or select Auto which scales such that the entire batch layout can be seen on the screen (see previous page).

In this view, FlowJo is drawing four columns of Frames. This option is controlled by the ***Layout Specification*** popup menu, immediately to the left of the Help button. Here you can select any number of columns between 1 and 4, or, you select Custom... and define exactly how you want FlowJo to arrange the frames on your page.

You will notice that FlowJo draws gray lines in the window: these correspond to page breaks were you to print the document. (Select a small magnification, like 12.5%, to see many pages at once). Note that as you change the viewing magnification, the relative scaling of the graphs to the page boundaries do NOT change – you are not changing the print magnification!

Changing the print magnification (and orientation – landscape or portrait) can be easily done using **Page Setup...** from the **File** menu and setting the magnification you want. You can also specify that FlowJo automatically scale one page to be as wide as the graphic display. This is done by selecting Scale To Page in the popup near the bottom of the window (right). When **Scale To Page** is selected, the page magnification (but not orientation) from **Page Setup...** is ignored.

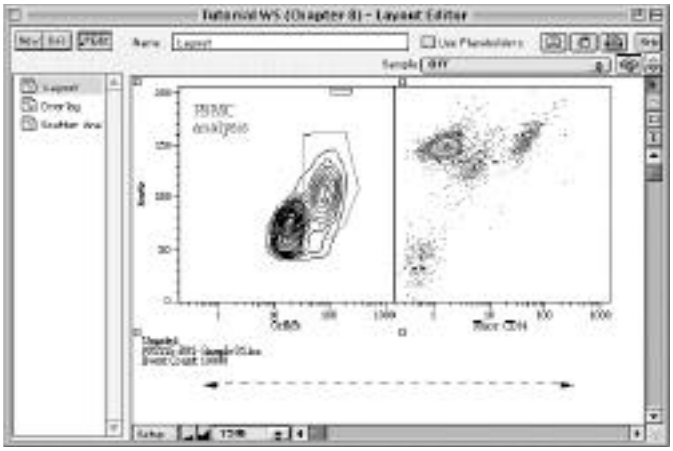


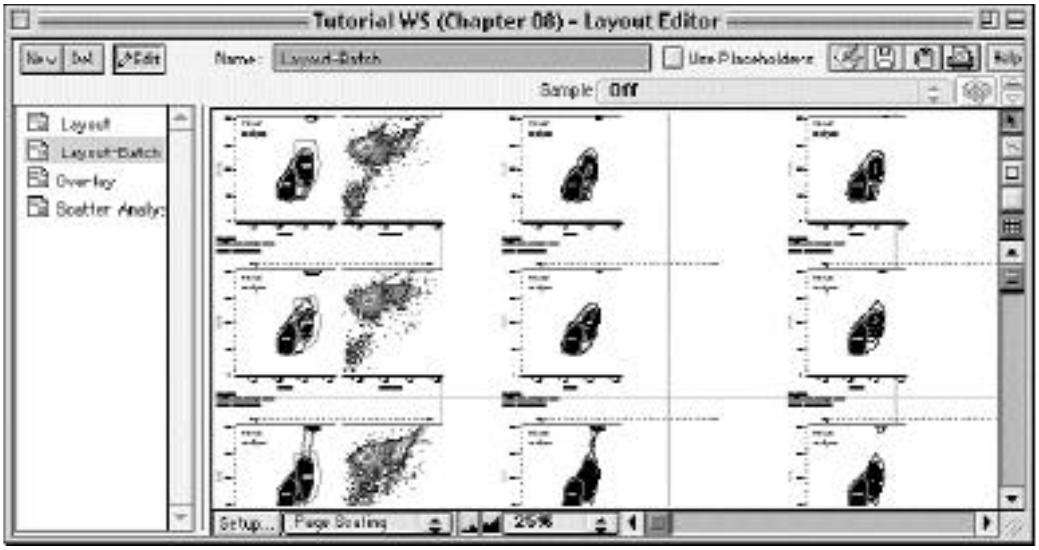
If you select Show Breaks, then FlowJo will rescale the page size to whatever you have selected with **Page Setup...** If you select **Avoid cut-offs**, then FlowJo uses your Page Magnification, but places frames on the page such that frames don't cross page boundaries (unless a frame is larger than a page).

Use the current drawing to experiment with the three options that affect how graphs will be printed: (1) the layout dimensions, using the Layout Specification popup menu near the top of the window; (2) the page dimensions, using **Page Setup...** ; and (3) Page break control, using the popup at the bottom of the window.

Once you have arranged the Frames exactly the way you want, you can print them – you will know exactly how they will appear on the pages!

It might be cumbersome to have to redesign the printing layout every time you create the batch output. Therefore, FlowJo lets you save the current state of the Batch Output with the Layout... simply select **Save Layout Settings** from the **Layout** menu (see right), or save the settings when the batch report is created. Either method saves the values of the **Page Setup...** magnification and orientation and the setting of the Layout Specification popup menu (i.e., how frames are to be placed in the overall output), the page break (and scaling) specification.





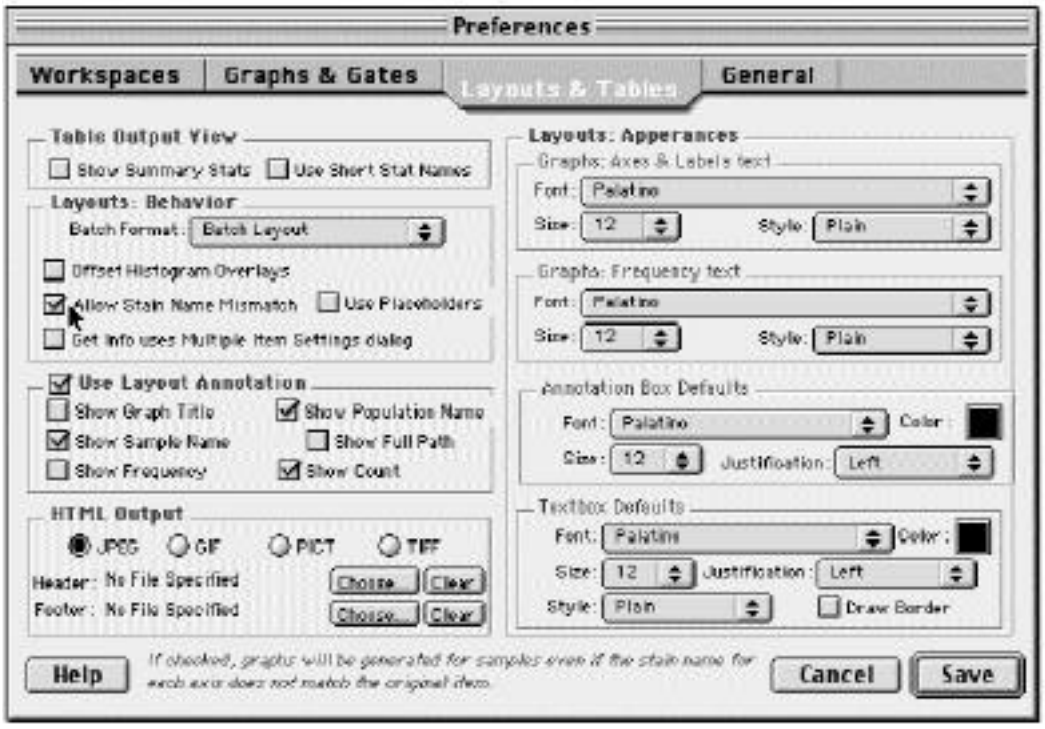
Close the ***Tiled Report*** window, and, in the ***Layout Window***, select the original "Layout" that has the two graphics (as shown at bottom of previous page). These two graphics are a forward vs. side scatter plot, and a FITC CD14 vs. Cy5PE CD45 plot. Click on the "Batch" creation button, and specify that you want to make a new layout (still four across). The new layout will be added to the list on the lefthand side. Its name is the original layout followed by the suffix -Batch. It should look something like that shown above, here called "Layout-Batch."

Now, each Frame consists of two plots, the arrow line, and the text item. Remember that FlowJo creates a new Frame for every sample in the current group. However, you may notice (depending on your Preferences setting) that most Frames only contain one plot! Why? Because the second plot requested a "FITC CD14" vs. "Cy5PE CD45" graph. Most samples in the current group were not stained CD14 and CD45; therefore, FlowJo assumes that this graph is irrelevant for that sample.

In some cases, however, you will want to generate the FITC vs. Cy5PE graph irrespective of what the actual stains were. FlowJo lets you over-

ride the stain specification. Select **Preferences** from the **Edit** menu, and click on the **Layouts** tab. FlowJo shows you the following window that sets a variety of preferences related to Layout generation: Click on the checkbox marked **Allow Stain Name Mismatch**. Save the preferences, and then regenerate the same layout. You will now see the pair of graphs shown for every frame.

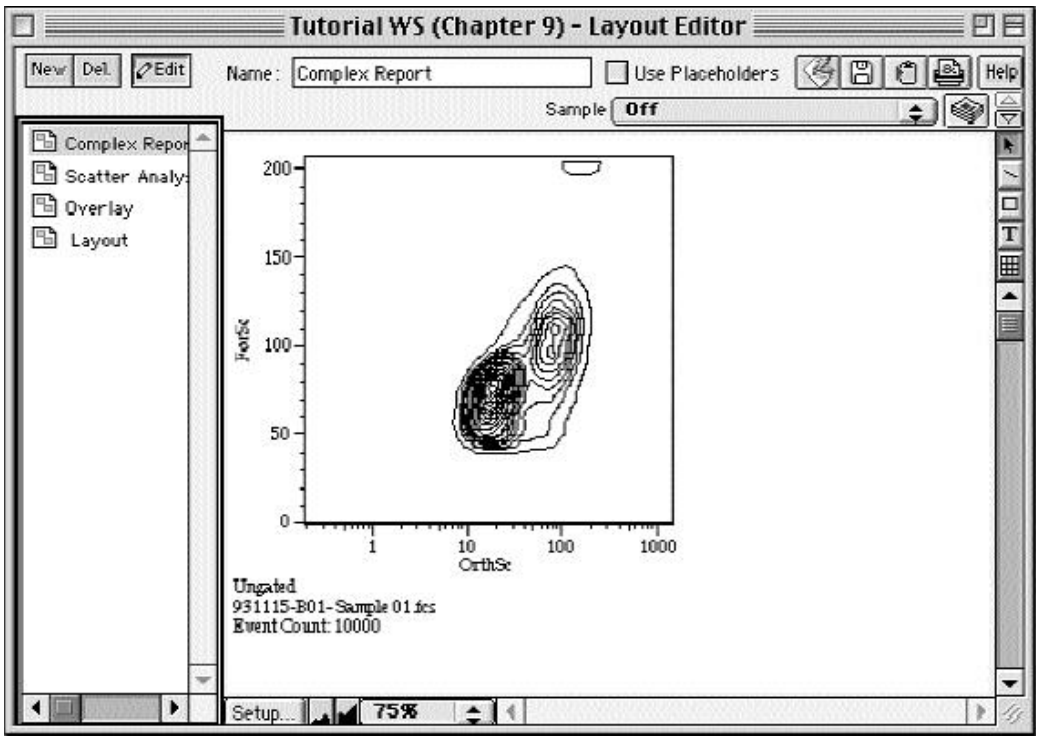
One last important piece of information. The ***Layout Editor*** view is ***live*** in that whenever you change a gate or analysis that might affect the view, the view is automatically updated to reflect the change. However, the ***Tiled Report, Web Report*** and ***Movie*** are NOT live. If you create a one of these reports and then change a gate, the **report is** NOT UP-DATED. You will have to regenerate the ***batch output*** to reflect the change you have made.



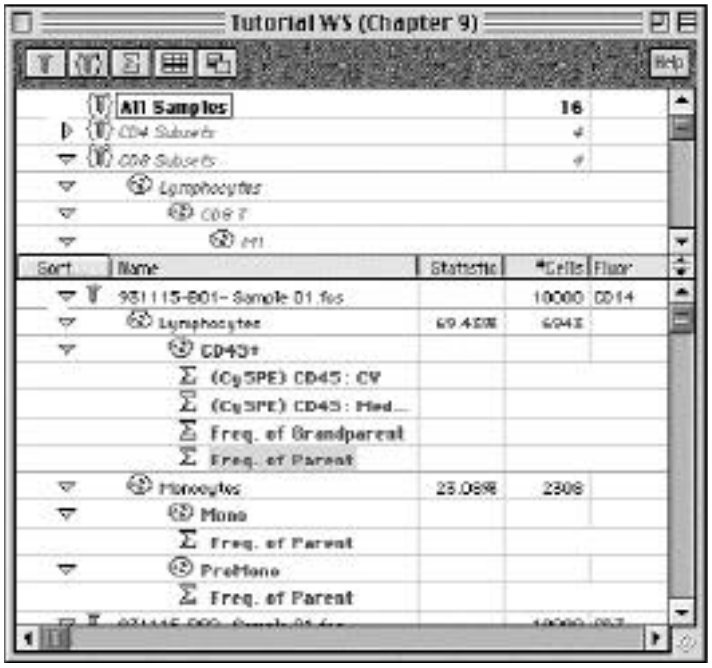
Lesson 9: Generating Complex Batch Analysis

In this chapter, you will build on what you learned in Chapter 8 so that you can generate graphical reports that include Statistics, as well as graphical reports that draw graphs from multiple different tubes for each frame.

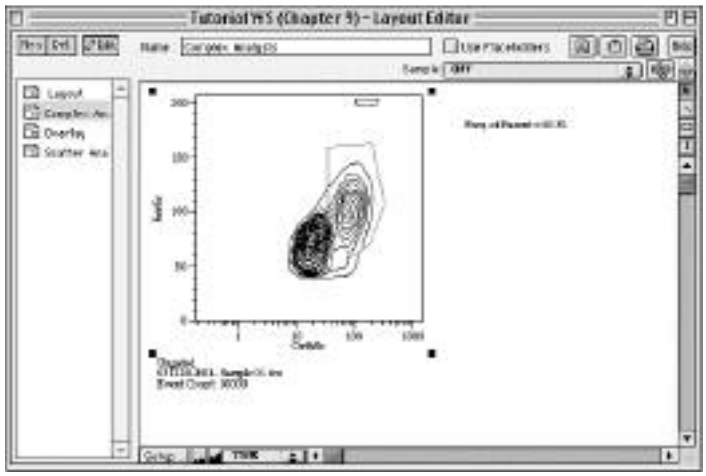
This lesson builds on the Workspace you have finished from Chapter 8; alternatively, you can open the workspace named "Tutorial WS (Chapter 9)". Open the Layout Editor , and create a new Layout named "Complex Report." Drag into the Layout View the first ungated sample, "931115-B01...". The Layout View should appear as below:



In the ***Workspace***, click on the **Freq. of Parent** statistic under the "CD45+" gate (right).



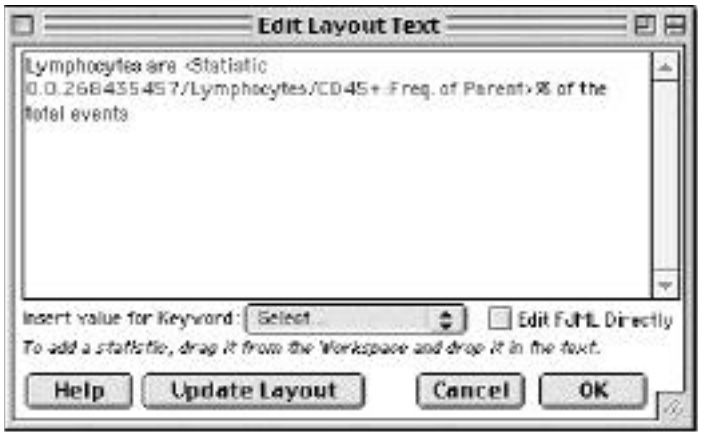
Drag this statistic into the ***Layout Editor***, holding the mouse button down as you move the statistic into the ***Layout Editor***, and drop it (releasing the mouse button) to the right of the graphic. FlowJo creates a text box, the contents of which is the name of the statistic and the statistic value, as shown in the figure:



You can add additional annotation to the layout, if you need to. Here we want to add some additional text to this box. To make room, make the text box a little bigger by clicking on the lower right handle, and resizing it to a larger size. Then, double-click on it to start editing. FlowJo opens a new

Edit Layout Text Window, as shown to the right:

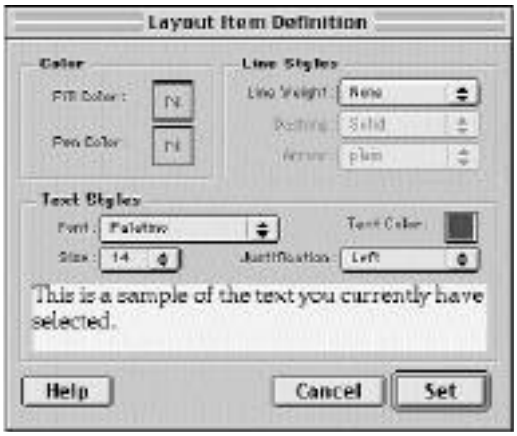
Note that the statistic value has been expanded to a bracketed ("<", ">") command (displayed in red). The command within the brackets tells FlowJo exactly how to get the desired statistic (i.e., which subset and which statistic is desired). Do NOT edit the text within the brackets, or else FlowJo may not be able to fetch the statistic value you want! (FlowJo won't let you edit the command unless you check the box, **Edit FJML Directly**) However, you are free to edit any of the text outside of the brackets. In this example, select all of the text in front of the first bracket and change it to "Lymphocytes are ", and after the second bracket, add the text "% of the total events". The window should look like this:



You can click on the Update button to see the changes to the Layout View, or click on **OK** to accept changes and go on. You can leave this window open in order to drag other statistics into the text (See Chapter 10). Note that you can also select keyword values to add to text boxes (the keyword values are data encoded in the file itself). You can create additional text boxes with more statistics by dragging them from the workspace. You can also add

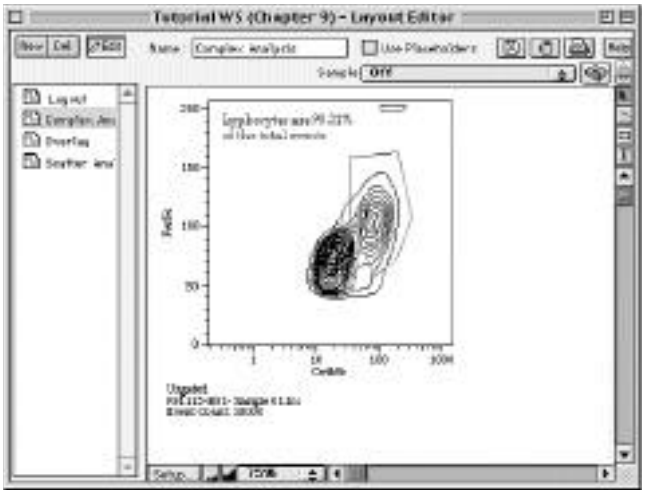
multiple statistics to one text box: either drag another statistic into an existing text box, or shift-click multiple statistics and drag them into the Layout View (they are all appended into the same text block). Again, you are free to edit the text outside of the brackets; don't edit text within the brackets.

If you click on the text box with the statistic, and select **Get Info...** from the **Layout** menu, you will see the ***Layout Item Definition*** window, from which you can change the particulars of the text display (shown here).



Select a red text color, and 14 point font size; click **Set**. Now click on the text box, and drag it so it overlays the graphic item in the **Layout View**. The view should look something like what is shown below.

Note that, like the graphs, statistics in the Layout editor are also ***live***. If gates change, causing the number of events to change, then they are automatically updated. In addition, Statistics respond to the ***Iteration value***; if you select a specific sample to view, then statistics are shown for that sample. (If you select a statistic of a subset that does not exist in the current Iteration sample, then



the statistic value is shown as "n/a". Likewise, a graph for such a subset would be shown as a Placeholder.)

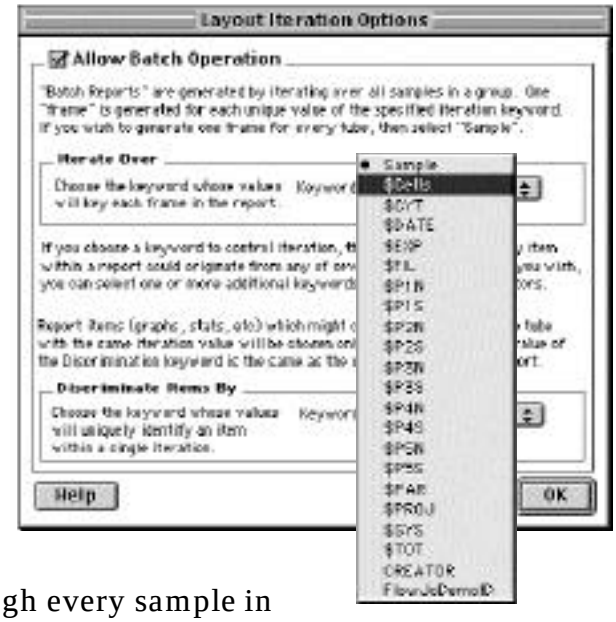
The final topic in this Chapter deals with Layouts that derive graphs (or statistics) from different samples (tubes). This will only be useful if your data files are properly annotated!

As you learned in Chapter 8, FlowJo forces all graphs to be derived from the same sample during iteration. To create a batch output, FlowJo forces the iteration value to cycle through all possible values (for the current Group). By default, this means one Frame for every sample (tube) in the Group. However, you may want to generate graphic reports wherein each Frame derives graphics from multiple samples. For example, you may want to generate one frame for each patient, and therefore iterate by patient ID. Alternatively, you may want to iterate such that each iteration value corresponds to a different tissue studied from an animal, where you have performed multiple stains on the tissue samples. In these cases, you would like to Iterate not over samples (or tubes), but to iterate over Patients or Tissues.

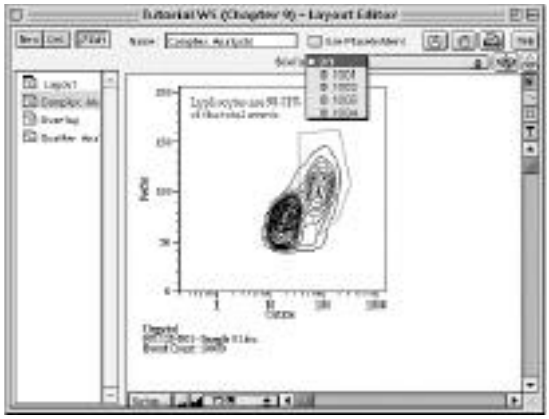
If your FCS data has keywords that contain such information, then FlowJo gives you this ability. If you don't know how to add this information to your data, ask your local Flow Cytometer operator or Instrument Sales Representative. For the demonstration in this tutorial, the data supplied has a Keyword Field, $Cells, which has a unique value for each different Patient sample. In the demonstration data, there were four patient samples (ID0001 to ID0004) that were stained with four different combinations of antibodies. (The Experiment 2 Folder has Patient ID0005 through ID0018; you may later wish to add that data to play with a larger data set).

We will now tell FlowJo that it should iterate over patient samples. To do this, select **Iteration Options** from the **Layout Menu** (see left).

FlowJo now shows you a window where you can specify which FCS keyword should be used as the controller for Iteration. By default, for new Layouts, this is set to **Sample**, meaning that each tube in the Workspace is a unique ***iteration value***. As shown in the figure, select $Cells, which, for this dataset, contains the unique patient ID.



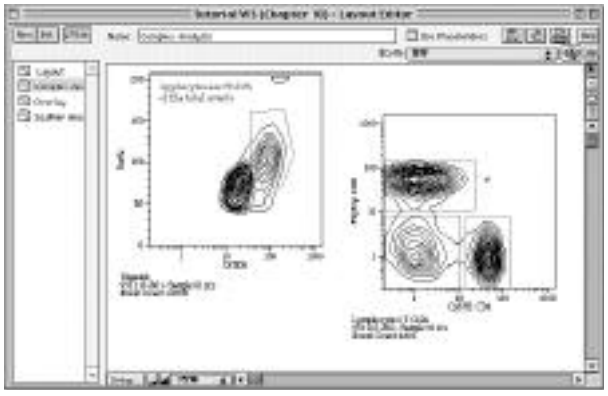
When you click on OK, FlowJo will now search through every sample in the current Group, and build a list of unique values of the Keyword **$Cells** found in the current group. This list is shown in the Iteration Popup menu; you can see it by clicking on the menu (shown here, at right).



Note that the title for the Popup menu has changed from **Sample** to **$Cells** to let you know that the Attribute for Iteration is now different. In the list of all of the samples in the current workspace, there were four unique values of **$Cells.**

When you now select any given value of the ***iterator***, then, for each graph and statistic in the Layout View, FlowJo searches through the current group to find the first sample that (1) has the Keyword value corresponding to

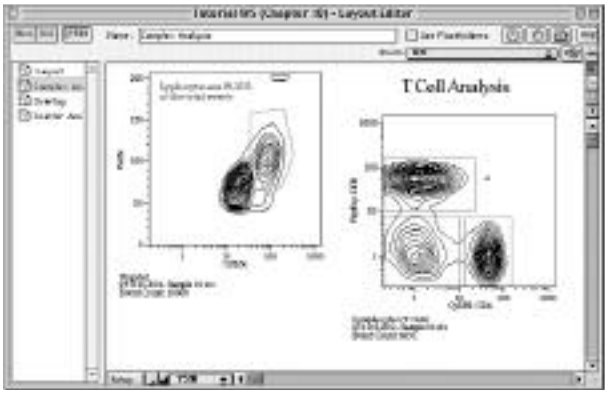
the current Iteration setting, (2) has the subset specified by the graph or statistic, and (3) has the appropriate parameters for the graph, and (4) has the correct stains defined for the graph. (Criterion #4 can be overridden by the Preferences, as explained in Chapter 8. However, you should in general not override this Preference if you are performing complex layouts like this; since your data is surely correctly annotated already!).



Set the ***iteration value*** back to **Off**. This specifies that FlowJo will only show you the graphs and statistics from the originally-dragged subset.

The graphs and statistics shown in the Layout View were derived from the first antibody combination (CD14/CD16/CD45). Now we will drag in a graph from a different staining combination. Select the "T cells" subset from the sample "931115-B02..." and drag it into the Layout Editor next to the first graph. Your **layout** should appear as above.

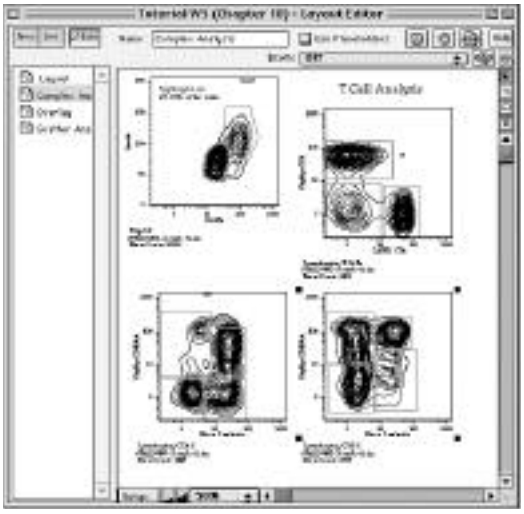
Add a text box item above the second graph, typing in "T Cell Analysis". Select the text box, then choose Get Info... from the **Layout** menu, and set the font size to 36 point. The Layout View will look something like that shown here:



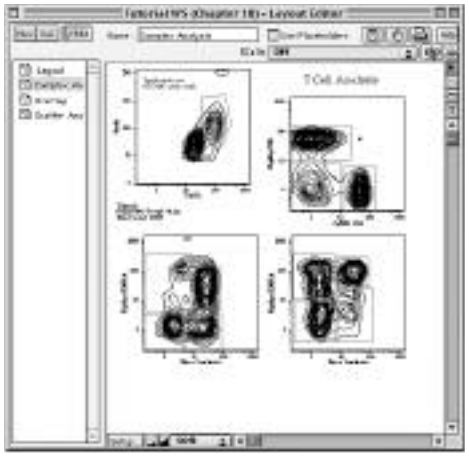
Make the ***Layout Editor*** Window larger (by dragging the lower right hand corner to resize the

window), in order to make room for more graphs in the Layout View. Back in the ***Workspace*** Window, click on the "CD4 T" subset and ***shift***-click the "CD8 T" subset under the next two samples (as shown to the right).

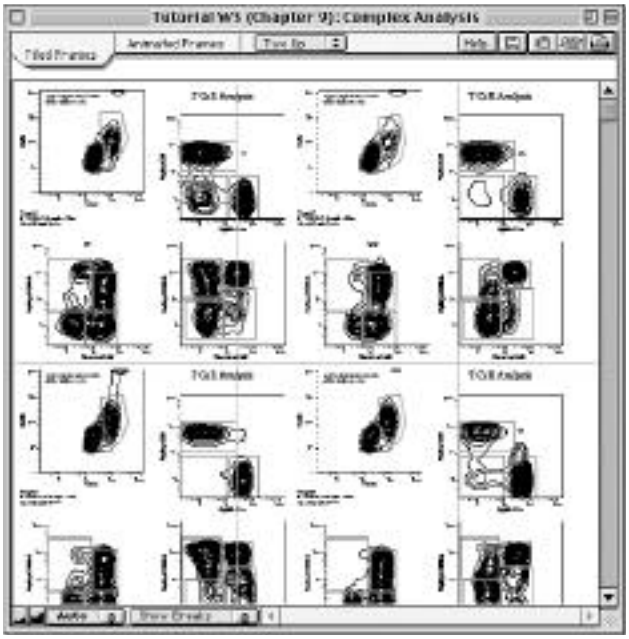
Click on one of these and drag (both will be dragged) into the Layout View, just below the first graphic. Now two more



graphs appear (below). Select the annotation text boxes under the three newest graphs, and press the ***delete*** key to remove the annotations. Move the graphs a little closer together (see right). Now you have created a graphical report that draws graphs from different tubes of the same patient. Click on the **Iteration** popup menu, and select "ID 1004". All four graphs and the statistic change, and now are drawn from the fourth patient sample in the workspace. You can select any patient sample



to view the graphs. To view the tiled report, just click on the ***batch generation button***. FlowJo now iterates (only four times!), and generates four ***frames***: one for each unique value of **$Cells.** You should see a window such as here:



You can quickly generate single page reports (one page per frame) by selecting 1-Up and **Scale To Page** from the appropriate popup menus inside the ***Stack View*** window. If you choose to print the ***Batch Output*** you will get four pages. Finally, as you learned before, if you want to save the Layout Settings, select the **Save Layout Settings** item under the **Layout** menu.

You may wish to experiment some more by dragging in the folder for "Experiment 2" on to the current workspace, and regenerate these layouts. They will be considerably more complex, as you will have 18 patient samples; however, you will quickly appreciate how little additional work is involved in your future analyses!

In the next Chapter, you will learn some additional Layout Editor techniques, including creating Grids and placing background graphics underneath the reports.

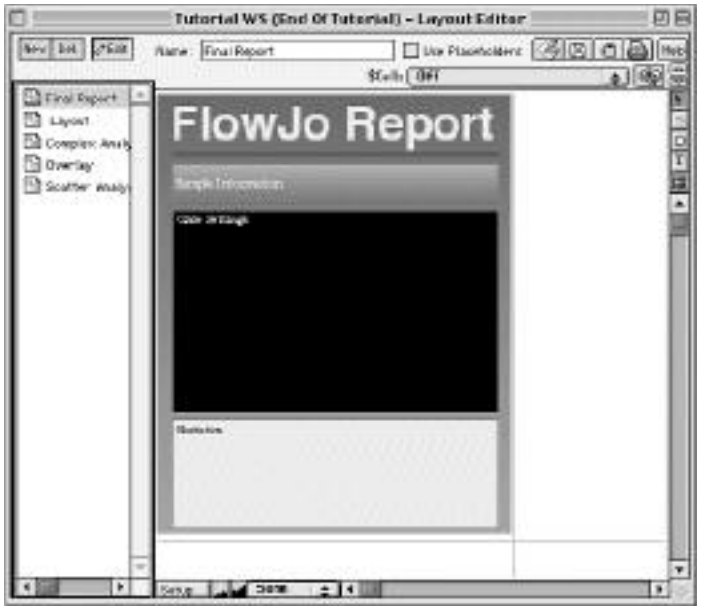
Lesson 10: Creating Finished Reports

In this chapter, you will learn how to use some of the advanced features of FlowJo's Layout Editor to create reports. You will learn how to create live text objects that contain sample-specific information and statistics, how to put in a backdrop containing (for example) logos or specialized forms, and how to manipulate Layout Grids–specialized tabular elements that can contain text, tables, graphs, or any other items.

This lesson builds on the Workspace you have finished from Chapter 9; alternatively, you can open the workspace named "Tutorial WS (Chapter 10)". Open the ***Layout Editor*** , and create a new Layout named "Final Report." Select "50%" in the scaling popup menu (at the bottom of the window) so that you can see the outlines of the page breaks.

First, add a backdrop. From the **Layout Menu**, select **Add Picture...**. Navigate to the Tutorial Folder, select "All Readable Documents" from the popup menu, and open the file "Report Backdrop" (see window below) This is a PICT file that was created using CanvasTM; you could open any PICT file generated by any program and add it to the layout. (You can also Copy and Paste any graphic from a drawing program into FlowJo's ***Layout Editor***).

FlowJo adds the PICT to the layout. This will be the template upon which you will add other elements (like statistics and graphics) in order to create the finished report. The layout window should appear as shown here:



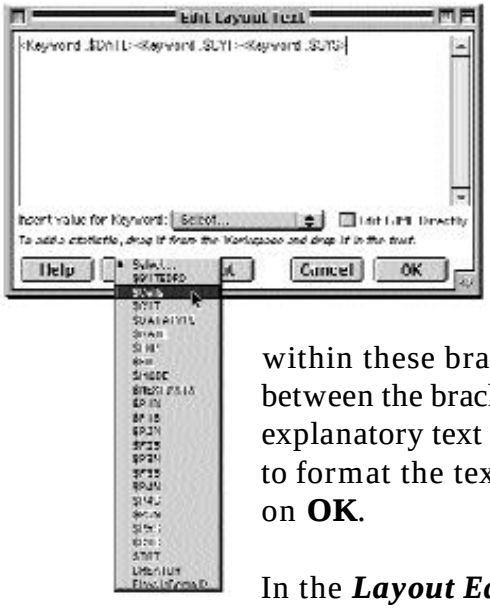
For your own reports, you can generate whatever design you wish – including specific areas for text, graphics, signatures, etc. Simply design the form in any graphics program and paste it into the ***Layout Editor*** , or save it as PICT and open it as you did this one. (Note: the printable area in FlowJo's layouts is approximately 7.6 x 9.5 inches at 100% Page Setup).

In this report, you will include three separate elements. In the top panel you will create a text box containing some information regarding the sample. In the second panel, you will put graphs that show the gating scheme used; finally, the bottom panel will contain statistics and graphs.

Because this layout will draw on information from multiple tubes for the same individual, you will need to set the Iteration Options to **$Cells.** (If you don't remember how, see the second half of Chapter 9.)

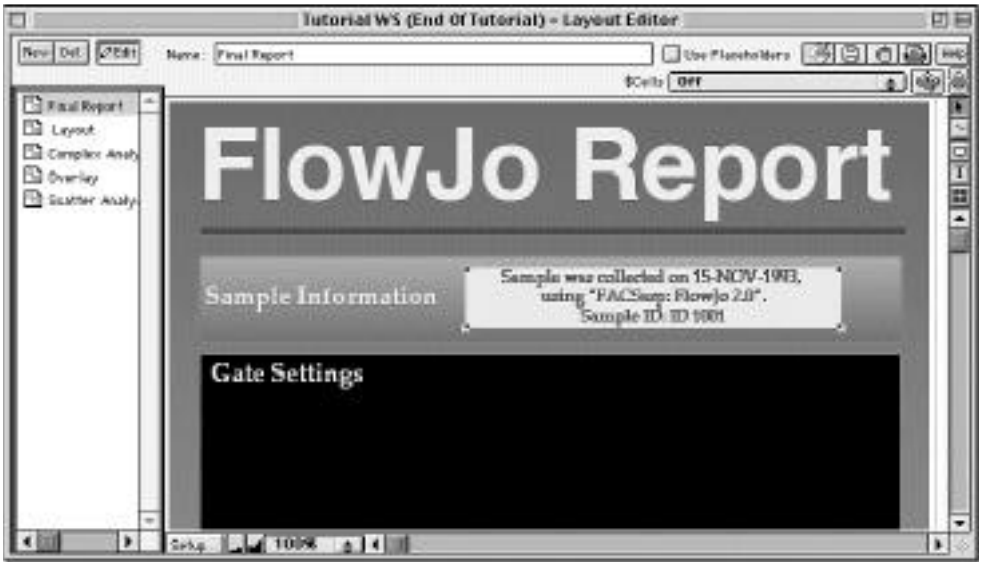
Begin the first panel by clicking on the text box tool (the T on the right side of the window), then click-and-drag a rectangular area that will fit to the right of the "Sample Information" text and within the gray area. FlowJo brings up the ***Text Box Editing*** window. Here you will select

several keywords that contain sample-specific information for entering into the text box. To insert a keyword value, simply select it from the popup menu (as shown). Begin by selecting the keywords **$DATE** (date of sample collection), **$CYT** (cytometer used for collection), **$SYS** (the system on which data was acquired), and $Cells (the sample identification). In your own datasets, you may have other keywords with important information that you can add.



Each keyword command is bracketed by "<" and ">"; do not edit any text within these brackets. However, you can add text between the brackets. As shown below, you can type in explanatory text (and hit return to create line breaks) to format the text box. When you are finished, click on **OK**.

In the ***Layout Editor***, magnify the view to 100%. Click on the text box you have just created, and select **Get Info...** to change the text style. For this example, set the background color to gray, the text color to blue, and the text format to bold and centered. (Note that the Pen Color applies to the line drawn around the text box, should you choose a **Line Weight** other than None.) When you are finished, click on **Set**, and the ***Layout Editor*** should look

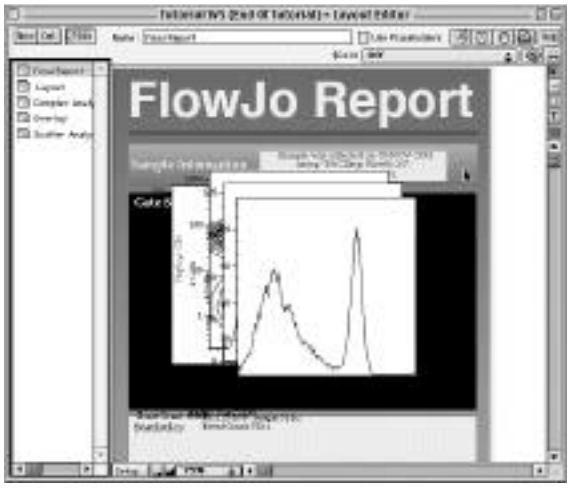


something like that above. If you wished to have a particular keyword value in a different font or color, you would have to make a separate text box for it and format it accordingly.

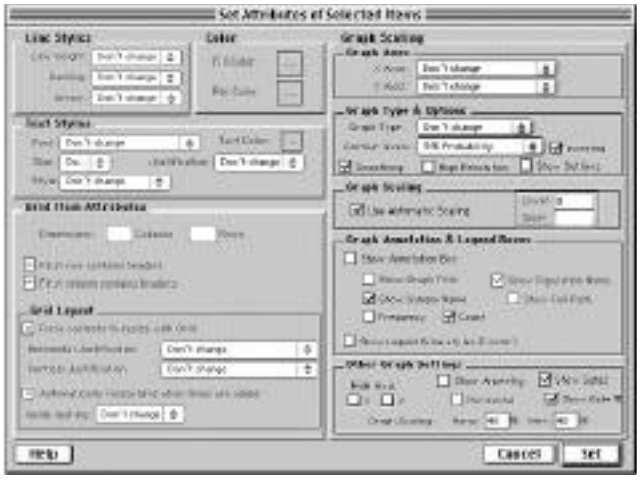
The next job is to add graphics into the middle panel that show how each antibody staining panel was gated. This is similar to some of the layouts you created in previous chapters. First, drag the ungated sample from the Workspace into the ***Layout Editor***, dropping it over the Gate Settings panel. You will note that you cannot see the text, because the graphic text is black. We will change this momentarily.

However, first drag in three other graphs into the ***Layout Editor***: as shown above, select the "T Cells" graph from the second sample, and the

two “Lymphocytes” gates from the CD4 & CD8 staining panels. ***Shift***-click these and drag them all at once onto the ***Layout Editor***. Your view will probably look something as shown here.

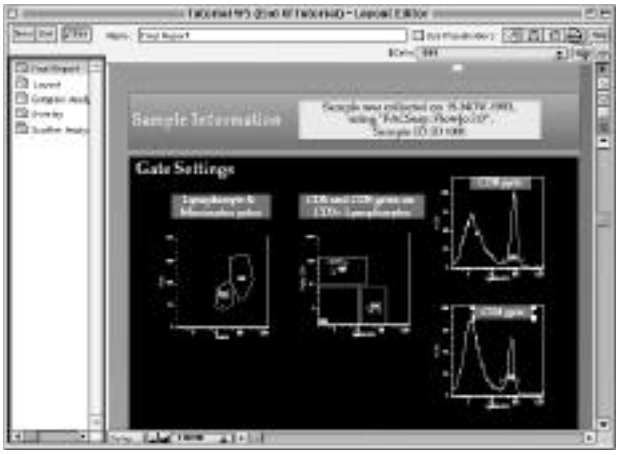


You will now change the attributes on these four graphs all at once (to save time). Select all four graphs (you can use a ***Marquee Selection*** by clicking outside the bounds of all four and dragging to include them all, or simply ***shift***-click all four). Double-click on the selection (or select **Get Info...** from the Layout menu). FlowJo shows you a display that lets you set attributes for all selected items at once.



As shown in this window, change several attributes. First, check the Inverted check box in the Graph Type options. Next, click on Show Annotation Box twice (a check mark means that the attribute is “On”; a blank box means the attribute is “Off”; and a dash means that some items have the attribute on, others have it off, and the setting should not be changed for any of them). Also check the boxes for **Show Gates** and **Show Gate %**, and type in values of “40” in the two scaling boxes near the bottom. When you are done, click on the Set button. All four items are changed as you requested.

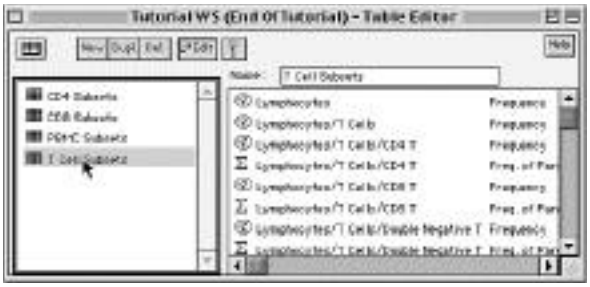
Now move the graphs into different locations, perhaps as shown in the example on the right (here, the lymphocyte & monocyte gates are shown first, then the CD4 & CD8 gates on the CD3 subset, and on the right, the CD4 & CD8 gates). Note that each graph is derived from a different tube. The graphs may appear very dim at low magnifications; however, they will print just fine (and, if you scale up to 200% or greater, you can see that they are drawn fine at high resolution).



Finally, you can create some text boxes to annotate the graphs. In this example, the new text boxes were created with a white text overlaying a dark-blue background. Tip: create one text box, format it exactly how you want. You can then duplicate it (using copy-paste, or holding down the ***option*** key while you click-and drag that item). Double-click on the text box to change its contents.

For the final panel, we will create two different tables. The first table will have just statistics; the second table will mix statistics and graphs.

The easiest way to create a table in the ***Layout Editor*** is to drag a table from the ***Table Editor*** and drop it in the ***Layout Editor***! Open the Table Editor window, and click on the table you previously created, "T Cell Subsets. " Now, drag this table



onto the ***Layout Editor***, letting go of the mouse when it is over the gray "Statistics" panel. FlowJo automatically creates a Grid item, filling it with information & statistics from the ***Table Editor.***

In FlowJo, a Grid Item is simply a collection of containers that hold other Layout Items. In this case, the Grid Item contains a series of Text box items, each with either static text (titles), or with Statistic commands. Click on one of the boxes in the grid (it will be highlighted in light blue), then double click on the text. You can edit it however you wish, just like a regular text box.

In this Grid, the fourth column ("For") is unused, because none of the statistics here apply to a specific parameter (like forward scatter or fluorescence). We can delete this column: click on any cell in the fourth column, and press the ***delete*** key. FlowJo asks whether you wish to delete the contents of this cell, or the row or column containing the cell. Click on **Delete Columns**.

The next order of business is to reduce the font size in the table items so that they fit better. Click on the top-left cell in the Grid, and ***shift***-click on the bottom-right cell in the Grid: this will select the entire range of cells including these two–i.e., all cells in the table. Double click on any cell with text in it, and you will see the window to set attributes on multiple items simultaneously. As shown here, select a font size of 9, and

click on Set. Next, resize the Grid to make it smaller. Click on the lower right hand selection handle and drag it to make the table fit in the area of the ***Layout Editor.*** You can resize individual rows and columns by clicking on any dividing line and dragging. Resize the columns so that the full text is shown in each cell (see top right).

Finally, while the Grid is still selected, choose **Get Grid Info...** from the **Layout** Menu. Here you can select specific attributes about the Grid. Set the Inner Dashing to **Short Dash**, the fill color to a light Yellow, and the Pen color to dark blue. Click on **Set** when you are done. (For more information about other Grid attributes, you can click on the **Help** button).

If you increase the magnification on the ***Layout Editor***, you should see your table similar to the one show here.

Now it's time to create the other Grid. In this Grid, you will

have three rows: one title row, one for CD4 T cells, and one for CD8 T cells. As well, you will have 4 columns: the first is a title, the second will have the Median CD3 fluorescence on the specified subset, the third will have the percent of CD3 T cells that are CD4 (or CD8), and the fourth will have a histogram of the CD3 intensity for the gated subset.

To create a blank Grid, click on the Grid tool (it is the one right above the vertical scroll bar at the edge of the Layout Window). Click and drag a rectangle that occupies the right portion of the Gray Statistics panel of your layout view. (You may have to adjust the magnification in order to see the full area of interest). FlowJo now asks you what dimensions to assign to the Grid; enter 3 rows and 4 columns.

FlowJo now shows the empty Grid placed in the ***Layout Editor*** (see below, left): Select the Grid, and choose **Get Grid Info...** from the **Layout** menu. Select a light yellow Fill Color, **Dotted** for the Inside Dashing, choose that the first row and the first column have headers, and, most importantly, select the option to **Force Contents to Resize** With **Grid**, and unselect the option to **Automatically Resize Grid when Items are Added**. Unchecking this last option means that FlowJo will not resize

the Grid when you later add graphs or text boxes to accommodate them. Further, the Force Contents to Resize With Grid option means that when items are added, they are automatically resized to fit within the Grid.

Finally, it is time to start annotating the Grid contents. If you option-double-click on any empty cell, FlowJo automatically creates a Text Box in that cell. Use this feature to add the titles Population, Median CD3, % of CD3, and CD3 Histogram to the first four cells in the top row. Add the titles CD4 cells and CD8 cells to the second and third cells of first column.

Select all of the cells in the Grid, and change the text attributes to a 9-point font with Bold text. Resize the columns and rows to make the Grid look better. Your ***Layout Editor*** may appear as shown here.

Note that any item in a Grid can be move out of the Grid or to another position: just click on the item (so the cell is selected), and drag it to a new position! Likewise, you can take any item from that Layout View, and drop it onto any empty cell in the Grid to make it part of the Grid. To add graphs and statistics to the Grid, you will select them from the Workspace, and drop them into the appropriate positions in the Grid.

First create the appropriate statistic. In the Workspace, select the "T Cell Analysis" Group. To a CD4 gate within the first sample, add the statistic "Median Fluor:CD3". Drag this gate to the Group's CD4 and CD8 gates

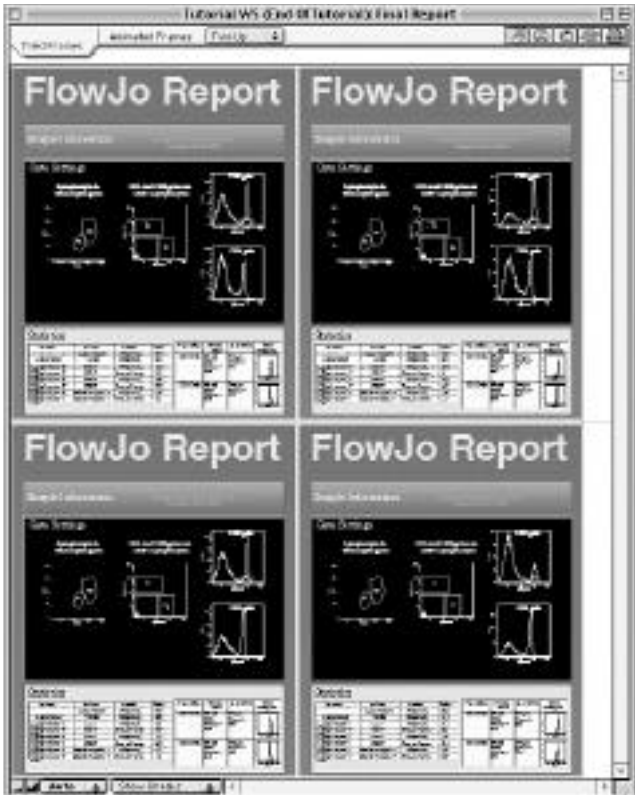
so that it is applied to all samples in the workspace. Your Workspace window should appear as shown here:

Now click on the Median CD3 statistic under the CD4 gate, and drag it to the second column of the second row of your new Grid (drop it when that cell is highlighted). FlowJo creates a new text box with the statistic and adds it to the Grid. Add the **Freq. of Parent** statistic to the next column. Repeat the two drags for the statistics bound to the CD8 subset into the third row of the Grid.

Select the four new Grid items, and change their font size to 9. If you wish, you can double click on each item individually (make sure that only that Grid cell is selected) in order to edit the text: remove the annotations from the Text to leave only the statistic for each cell.

To add graphs to the Grid, click on the population of interest (from the ***Workspace***) and drag it into the correct cell of the Grid (Or, if you already have the graph in the ***Lay - out Editor***, you can drag it and drop it into the Grid). Select the CD4 subset and drop it into the last column; likewise for the CD8 subset. To change the graphs to histograms of CD3, select the two cells with graphs, and double-click. Change the X-Axis to "Fluor" and the ***graph type*** to **Histogram**. At this point, your ***Layout Editor*** should look something like that shown above.

Now you are ready to generate the full report for all samples. Remember to select the All Samples group first (any batch layout operation is only performed on the current group), and click on the ***Batch*** button. FlowJo compiles all of the graphs, statistics, text, grids, and backgrounds and puts them into a single window (see below). From here you can print a report, publish to the web, or copy to slide-presentation programs.



Most importantly, your layout is now ready for the next experiment: all you have to do is add the samples to the ***Workspace*** and run the Batch processor: the report will be generated without having to re-generate the layout template! And you can have as many of these templates as you wish for any ***Workspace***.

This ends the tutorial lessons. There is more documentation available in the reference web pages, which you'll reach from any of the ***Help*** buttons within the program, by typing ⌘-? anywhere in FlowJo, or by looking at:

<http://www.flowjo.com/v3/reference.html>.

If you have any questions, or ideas for improvements, please contact us at: flowjo@treestar.com.